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(54) **Genetic detoxification of pertussis toxin**

(57) A new method is described for the preparation of a safe, immunogenic and efficacious vaccine for protection against the disease pertussis. In development of this vaccine, specific functional sites of pertussis toxin have been identified, and using this information, defined mutant holotoxins have been produced by site directed mutagenesis of the toxin gene. A number of these toxin analogues are detoxified, retain an immunodominant S1 epitope, are immunogenic and are protective in the standard pertussis vaccine potency test in mice. Mutant strains of B. pertussis are described from which the native toxin operon has been deleted and replaced by a mutant gene which expresses the mutant holotoxins. The native TOX operon of a specific B. pertussis strain has been isolated, sequenced and cloned into plasmid DNA.

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## FIELD OF INVENTION

The present invention relates to a novel method for the detoxification of pertussis toxin by the genetic manipulation of DNA segments coding for one or more amino acid residues essential for the toxin's biological activity. It also relates to a procedure for the creation of genetically altered Bordetella pertussis bacteria that produce the said detoxified pertussis toxin.

## BACKGROUND OF THE INVENTION

Whooping cough, or pertussis, is a severe, highly contagious respiratory disease of infants and young children caused by infection with Bordetella pertussis. Owing to the many virulence factors associated with this organism, the pathogenesis of the disease is still not fully understood; however, it is generally recognized that the major systemic effects are caused by pertussis toxin (PT). This material exhibits a wide range of biological activities as illustrated by such alternative names as lymphocytosis-promoting factor, histamine-sensitizing factor and islet-activating protein. Many of these effects are associated with its biochemical function as an adenosine diphosphate (ADP)-ribosyltransferase. ADP-ribosylation of certain acceptor guanosine triphosphate-binding proteins leads to a loss of control over a variety of metabolic pathways mediated by cyclic adenosine monophosphate and by phospholipase C. In the absence of a protein acceptor, PT also catalyses the hydrolysis of nicotinamide adenine dinucleotide (NAD glycohydrolase activity).

Conventional killed whole-cell pertussis vaccines contain a mixture of antigens and there has been a great deal of work towards the development of a defined acellular vaccine comprising specific protective antigens. PT is the most significant protective antigen. Other antigens under consideration are agglutinogens and filamentous hemagglutinin (FHA).

Normally PT and other antigens are chemically inactivated, or toxoided, using agents such as formaldehyde, glutaraldehyde or hydrogen peroxide. This approach has the serious disadvantage that a delicate balance must be sought between too much and too little chemical modification. If the treatment is insufficient, the vaccine may retain residual toxicity owing to the presence of a small proportion of unchanged virulence factors including PT. If the treatment is too excessive, the vaccine may lose potency because its native immunogenic determinants are masked or destroyed. This problem is of particular concern in the case of PT, since the catalytic subunit is comparatively difficult to inactivate by aldehydes. The possible residual toxicity or reversion of toxoided whole-cell pertussis vaccines has been questioned for many years, and it has been suggested that in rare cases the vaccine might cause major neurological damage. All pertussis vaccines that are in use at present, or in the trial stages, depend on the inactivation of the antigens by chemical means, which introduces the problems previously mentioned. It is obvious that if an inactivated vaccine could be designed without resorting to the toxoiding process, but preserving the native structure of immunogenic and protective epitopes, an additional degree of safety and efficacy would be added. For these reasons the inventors have genetically manipulated the gene coding for PT, and constructed strains of B. pertussis that secrete non-toxic PT analogues.

In its structural organization, PT belongs to the family of ADP-ribosyltransferase bacterial toxins, which also includes diphtheria toxin, Pseudomonas aeruginosa exotoxin A, cholera toxin and Escherichia coli heat labile toxin. Accordingly, it consists of two functional moieties: an A portion, which carries the enzymic activity, and a B portion, which binds to the host cell and permits translocation of the A portion to its site of action. In PT, the A portion is a discrete subunit, commonly denoted S1. The B portion is a non-covalent oligomer of five polypeptides arranged as two dimers, comprising subunits S2 plus S4 and subunits S3 plus S4 respectively, held together by a joining subunit S5.

The amino acid sequence of the S1 subunit reveals several features of interest. There are only two cysteine residues which form an intrachain disulphide bond; however, it is known that for enzymic activity the toxin must be reduced (Moss et al., J.Biol.Chem. 258, 11872, [1983]), indicating the importance of these residues. There are two tryptophans in S1, and it has been suggested that tryptophan residues are close to the NAD binding sites of diphtheria toxin and P. aeruginosa exotoxin A. Two conserved regions in S1 are also found in the amino acid sequences of cholera toxin and E. coli heat labile toxin (Locht & Keith, Science, 232, 1258, [1986]). In addition the NAD active sites of diphtheria toxin and P. aeruginosa exotoxin A have been shown to contain a glutamic acid residue (Carroll & Collier, Proc. Nat. Acad. Sci., U.S.A., 81, 3307, [1984]; Carroll & Collier, J.Biol.Chem., 262, 8707, [1987]).

As noted above, the B portion of PT mediates its binding to cellular receptors and contains two dimers. Whether each of these dimers bears a binding site remains controversial. However, the S2 and S3 subunits are similar in amino acid sequence and binding studies have indicated that lysine and/or tyrosine residues

of S3 in particular are implicated in the interaction of the toxin with its receptor. (Nogimori et al., Biochem., 25, 1355, [1986]; Armstrong & Peppler, Infect. Immun., 55, 1294, [1987]).

Site-directed mutagenesis of diphtheria toxin and P. aeruginosa exotoxin A at the NAD-interacting glutamic acid residues has led to significant reduction in ADP-ribosyltransferase activity (Tweten et al., J.Biol.Chem., 260, 10392, [1984]; Douglas & Collier, J.Bacteriol., 169, 4967, [1987]). Complete truncated forms of S1 and S2 have been expressed in E. coli (Locht et al., Infect. Immun., 55, 2546, [1987]). Mutations of the PT operon generated by transposon insertion, gene truncation or linker insertion have been introduced by allelic exchange into the chromosome of B. pertussis (Black et al., Ann. Sclavo, 175, [1986]; Black & Falkow, Infect. Immun., 55, 2465, [1987]). However, the biological and immunoprotective properties of fully-assembled recombinant holotoxins specifically detoxified by site-directed mutagenesis of functional amino acid residues have not been reported. The generation of such PT analogues for inclusion in a safe and efficacious pertussis vaccine is the subject of this invention

In testing for the efficacy and toxicity of materials that could be candidates for a protective vaccine, there are a number of in vivo and in vitro assays available. The standard test for potency is the mouse protection test, which involves intra- cerebral challenge with live B. pertussis. Newer vaccine tests measure the production of protective antibodies. A common toxicity test is the CHO (Chinese hamster ovary) cell clustering assay, which reflects both the ADP-ribosyltransferase and binding ability of the toxin (Burn et al., Infect. Immun., 55, 24, [1987]). A direct test of the enzymic activity of PT is the ADP-ribosylation of bovine transducin (Walkins et al., J. Biol. Chem., 260, 13478, [1985]).

## SUMMARY OF INVENTION

In accordance with the present invention, there is provided a novel method of detoxifying PT, which does not suffer from the drawbacks of the prior art chemical methods and yet provides an detoxified PT that retains its immunological properties without possessing undesirable side effects. In the present invention, amino acid residues of the toxin that are crucially important to its functional and toxic activities are identified. These residues are subsequently removed or replaced by site-directed mutagenesis of the isolated toxin gene. The mutated toxin operon resulting from such manipulations then is substituted for the native gene in the organism, which thereby produces the decreased toxicity toxin under normal growth conditions. In this manner, the three-dimensional structure and thus the immunogenicity of the PT analogue is minimally impaired. Indeed, an appropriate mutant form of the toxin on its own may provide satisfactory protection against the severe symptoms of pertussis, though other components may be required to establish resistance against the bacterial infection itself.

In accordance with one aspect of the present invention, therefore, there is provided an immunoprotective genetically-detoxified mutant of pertussis toxin. By the term "genetically-detoxified" as used herein is meant a pertussis toxin mutant which exhibits a residual toxicity of about 1% or less, preferably less than about 0.5%, of that of the native toxin. The residual toxicity is determined by CHO cell clustering assay and ADP-ribosyltransferase activity.

In accordance with another aspect of the present invention, there is provided a vaccine against Bordetella pertussis comprising an immunogenically-effective amount of the immunoprotective mutant of pertussis toxin or a toxoid thereof and a physiologically-acceptable carrier therefor. The genetically-detoxified pertussis toxin also may be used as a carrier protein for hapten, polysaccharides or peptides to make a conjugate vaccine against antigenic determinants unrelated to the toxin.

A further aspect of the present invention provides a method of production of the mutant, which comprises identifying at least one amino acid residue of the toxin which confers toxicity to the toxin; effecting site-directed mutagenesis of the toxin gene to remove or replace at least one such residue and to produce a mutated toxin operon; substituting the mutated toxin operon for the native gene in the Bordetella pertussis organism; and growing the transformed organism to produce an immunoprotective, genetically-detoxified toxin.

As will be apparent from the following disclosure, the present invention further provides novel strains of Bordetella pertussis from which the toxin operon has been removed or has been replaced by a mutant gene as provided herein.

## BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows the sequences of amino acids obtained by automated sequencing of radiolabelled peptides A and B and are compared with residues from mature S1;

Figure 2 shows the structures of various TOX clones obtained from the chromosomal libraries;

Figure 3 shows the construction of subclones containing the TOX gene from the genomic clone  $\lambda$  gtl1 15-4-1, with the TOX gene being inserted into the multiple cloning site of pUC8:2, which contains Bgl II and Xba I sites;

Figure 4 shows the construction of subclones of the TOX gene used for sequencing the operon. In (a), a restriction map of the TOX gene and the protein subunits are indicated, with clones being derived from the pUC8:2/TOX clone J-169-1, and the subunit genes being subcloned into M13mp18, M13mp19 or pUC8:2; as indicated; in (b), clones of the 5' region of pUC8:2, S1 in M13mp18 and S1 in M13mp19 clones are described; in (c), clones of S2 in M13mp18 and M13mp19 is shown; in (d), clones of S4/S5 in M13mp18 and M13mp19 are shown; and, in (e), clones of S3 and the 3' region in M13mp18 and pUC8:2;

Figure 5 shows the nucleotide sequence and structural gene translation products of the B. pertussis 10536 TOX gene;

Figure 6 shows the construction of TOX or TOX analogue genes in the broad-host-range plasmid pRK404 (Ditta et al., Plasmid, 13, 149, [1985]). In (a) and (b), there is shown the construction of primary TOX analogue genes in pRK404 from mutated genes and native genes, while in (c), there is shown a typical construction or a "crossed" mutant from two S1-mutated genes;

Figure 7 shows the development of a "suicide" plasmid, one capable of conjugative transfer but not replication, based on pRK404 and pMK2084 (Kahn et al., Methods in Enzymology, 68, 278, [1979]), for non-homologous recombination, the final plasmids also containing a Tn5-derived kanamycin resistance gene 3' to the TOX or TOX analogue genes;

Figure 8 shows the cloning of the 5'- and 3' flanking region of the TOX gene, (a) shows the construction of the 5' portion of TOX in pUC8:2 from the  $\lambda$  Charon 35 clone Ch421; (b) shows the construction of the 3' portion of TOX in pUC8:2 from  $\lambda$  Ch 111; and (c) shows the generation of a pUC8:2 clone containing TOX plus its 5'- and 3' flanking regions;

Figure 9 shows the construction of plasmids for the deletion of the TOX operon from the B. pertussis chromosome by homologous recombination; and

Figure 10 shows the construction of plasmids for reintegration of TOX analogues into the B. pertussis genome by homologous recombination, the final plasmids being based on the suicide plasmid shown in Figure 7 and containing the tetracycline resistance gene from pRK404 placed 3' to the TOX analogue gene.

## GENERAL DESCRIPTION OF INVENTION

It has been shown that the TOX operons from different strains of B. pertussis are nearly identical in sequence (Nicosia et al, Proc. Nat. Acad. Sci., U.S.A., 83, 4631, [1986]; Locht & Keith, Science, 232 1258, [1986]). The TOX locus is here defined as a DNA fragment beginning at the EcoR I cleavage site which encodes a 5'-flanking sequence, the promoter region, the structural genes for all PT subunits and a 3' flanking sequence. The TOX gene from B. pertussis 10536, which is the strain used by the inventors, was cloned and sequenced. Its nucleic acid sequence was found to be highly homologous to other published sequences, with four unique base differences downstream from the G of the EcoR I site defined as base 1. The complete nucleotide and corresponding amino acid sequences at the structural genes are shown in Figure 5.

The plasmid DNA of clone J-169-1 which contains the TOX gene from Bordetella pertussis 10536 cloned into pUC8:2 as a 4.6 kb EcoR I, BamHI fragment, has been deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland, U.S.A. on November 24, 1988 under American Number 40518.

The T at position 315 is unique to strain 10536 and there are three differences in the S1 gene at positions 710, 1200 and 1202, resulting in two unique amino acids, glutamic acid and valine, at positions 34 and 198 of the mature S1 sequence, respectively. The toxin genes of B. paraptussis and B. bronchiseptica are not expressed because of multiple mutations in their promoter regions, (Arico & Rappuoli, J.Bacteriol., 169, 2849, [1987]). This has allowed the use of B. paraptussis as a host for the expression of mutated toxin genes for screening purposes.

The inventors have shown that substitution of a single amino acid in S1, in particular at the active site for NAD hydrolysis (position 129), virtually abolishes the ADP-ribosyltransferase activity of PT. However, it may be desirable to alter several sites on the holotoxin to ensure complete safety. Accordingly, this invention applies to single or multiple mutations in both or either of the A and B portions of the toxin to abolish toxicity, and to the reinsertion of these mutations back into the genome of Tox<sup>-</sup> strains of Bordetella.

A number of strategies have been used by the inventors to determine regions of the toxin that might be closely associated with its biological activities, and, therefore, contain candidate sites for genetic manipulation.

PT was prepared from culture supernatants of *B. pertussis* (strain 10536). The crude solution was concentrated by ultrafiltration and passed through a fetuin-agarose affinity column to adsorb PT. PT was eluted from the washed column using potassium thiocyanate and dialyzed into a phosphate-saline medium. At this stage, the purity was 90-95%, as determined by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The major contaminant was FHA. Further purification was achieved by chromatography through a hydroxyapatite column, giving a material with a purity >99%.

The site of interaction of the S1 subunit with NAD was determined by photo-crosslinking NAD to isolated and purified S1 using [<sup>14</sup>C]NAD, labelled either in the nicotinamide carbonyl group or the adenine moiety. Radiolabel was efficiently absorbed from the nicotinamide moiety into the protein. The protein was then digested with trypsin and chromatographed on an HPLC column, giving two major radioactive peptides. After purification the two tryptic peptides were sequenced and the first fifteen residues corresponded to residues 118-132 of mature S1. In both the peptides, radioactivity was associated with an unidentified amino acid corresponding to position 129 in mature S1. Radioactivity was not detected in any other position. This established that GLU<sup>129</sup> is the site of photo-crosslinking of NAD and is therefore likely to be an important component of the nicotinamide interaction site. Significantly the sites of linkage in diphtheria toxin and *P. aeruginosa* exotoxin A are also glutamic acid residues and the three amino acid sequence commencing at GLU<sup>129</sup> of S1 strongly resembles the analogous sequences of the other bacterial toxins.

Chromosomal DNA was prepared from *B. pertussis* (strain 10536) and was digested with the restriction enzyme EcoR I in such a way that fragments were obtained ranging in size from a few hundred bases to a few kilobases. The DNA fragments were ligated with  $\lambda$  gtlI DNA which had been digested with EcoR I and dephosphorylated. The DNA was packaged into phage particles and maintained in *E. coli* Y1090 as a gtlI *B. pertussis* genomic library. Alternatively, *B. pertussis* chromosomal DNA was digested with the restriction enzyme Sau3A I to generate very large DNA fragments which were ligated with BamH I restricted  $\lambda$  Charon 35 DNA. The DNA was packaged into phage particles and maintained in *E. coli* LE392 as a  $\lambda$  Ch 35 *B. pertussis* genomic library.

These genomic libraries were plated and phage plaques transferred onto nitrocellulose filters. The filters were screened by DNA hybridization using an oligonucleotide probe specific for the PT S4 subunit. Positive plaques were further purified by two additional rounds of plating and hybridization. Phage DNA was prepared from the positive plaques and subjected to restriction enzyme digestion and Southern blot analysis. Clones containing the entire 4.6 kb EcoR I pertussis toxin operon (TOX) or portions thereof and with differing 5'- or 3'-flanking regions were characterized. The TOX gene was subcloned for sequence analysis and further genetic manipulation. Sequencing was performed using the dideoxy chain termination method and the results indicated four novel bases in the 10536 TOX gene as compared to published sequences.

Subclones of S1 or S3 genes in M13 phage were subjected to *in vitro* site-directed mutagenesis using the phosphorothioate procedure. Single-stranded DNA from those clones was annealed with oligonucleotide primers specifically designed to mutate or delete one or more amino acids. The mutagenesis was carried out using a kit available from a commercial source. Mutations were verified by sequencing of single-stranded phage DNA. Mutant subunit genes were recombined with the remainder of the operon to construct mutant holotoxin genes in the broad-host-range plasmid pRK404 maintained in *E. coli* JM109.

In order to characterize the holotoxin analogues, these plasmids were transferred to a spontaneous streptomycin-resistant *B. paraptussis* strain by conjugation on a solid surface, using pRK2013 as a helper plasmid. The colonies were selected on tetracycline-containing Bordet-Gengou blood plates. Mutated genes were also integrated into the chromosome of *B. paraptussis* by conjugative transfer of a suicide plasmid. The integration was either random or directed through homologous recombination utilizing the flanking regions of the *B. pertussis* TOX operon. Figure 7 shows the construction of a suicide plasmid containing mutants for random recombination.

Liquid cultures were grown in modified Stainer-Scholte medium containing methyl- $\beta$ -cyclodextrin in shake flasks (10 ml to 2L) or in fermentors (20L to 50L). The expression level of holotoxin analogues in culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA) and found to vary with the mutation. The residual toxicity of the analogues was measured by the CHO cell clustering assay.

A number of PT analogues were purified from 2L to 50L cultures of recombinant *B. pertussis* strains, according to methods described in detail for native PT. The ADP-ribosyltransferase activity of these mutants was determined as the extent of incorporation of radioactivity into bovine transducin from [<sup>32</sup>P]-labelled

NAD. Table 1a below lists the PT mutants generated and Table 1b below summarizes their residual toxicity and enzymic activity.

Selected purified mutants were tested in mice for acute toxicity, histamine sensitization activity and potency in the standard mouse intracerebral challenge test. These results are presented in Table 2 below and show that PT analogues have a markedly-decreased acute toxicity and histamine sensitization activity and that they are immunoprotective in the mouse potency test.

The immunological properties of PT analogues were further investigated by epitope mapping and by analysis of the antibody response in mice. Several monoclonal antibodies (MAbs) specific for individual subunits or dimers of PT were prepared and used to determine by ELISA whether the epitopes defined by these antibodies were affected by the mutations. The S1 epitope recognized by MAb PS21 is of particular significance.. since it is immunodominant in mice and this antibody confers passive protection in the mouse intracerebral challenge test. The preservation of this epitope in the PT analogues is indicated in Table 1b.

Immunogenicity studies in mice were performed on three purified PT mutants. Immune sera were tested for their ability to inhibit PT-induced CHO cell clustering (Table 3 below), and for their anti-PT, anti-SI and anti-B-oligomer antibody titres by indirect ELISA (Table 4 below).

To generate a B. pertussis strain expressing a mutated TOX gene suitable for vaccine production, the endogenous TOX operon was deleted by homologous recombination using electroporation of linear B. pertussis DNA containing the 5'- and 3'-flanking regions of the TOX locus. Selected mutant genes were then reintegrated into the TOX locus of the B. pertussis chromosome. Clones containing mutated TOX genes were grown and the culture supernatants assayed for level of expression of PT analogues and their residual toxicity as previously described. These results are shown in Table 5 below.

Certain Bordetella pertussis strains wherein the TOX gene has been removed entirely or has been replaced by certain clones, have been deposited with ATCC on November 24, 1988, as follows:

Strain	Modification	ATCC Accession Number
<u>B. pertussis</u> 29-9	TOX deleted (Tox <sup>-</sup> )	53838
<u>B. pertussis</u> S-2962-1-2	S1:GLY <sup>129</sup>	53837
<u>B. pertussis</u> S-3036-2	S1:GLU <sup>58</sup>	53835
<u>B. pertussis</u> S-3122-3-1	SA:ALA <sup>41</sup>	53834
<u>B. pertussis</u> S-3122-2-3	S1:GLY <sup>129</sup> , S3:ASN <sup>92</sup> ARG <sup>93</sup>	53833

The Tox<sup>-</sup> strain is a novel strain of Bordetella pertussis from which the toxin operon has been removed and from which foreign DNA is absent and which is capable of being grown in the absence of antibiotics to produce B. pertussis antigens free of pertussis toxin.

Each of the transformed strains is a strain of Bordetella pertussis in which the toxin operon has been replaced by a mutant gene formed by site-directed mutagenesis of at least one specific amino acid residue responsible for pertussis toxin toxicity.

The data presented herein demonstrate that the inventors have produced a series of pertussis toxin analogues that exhibit a substantial reduction in CHO cell clustering and enzymic activities (0.1 to 1% of the wild-type activity). Many of these analogues also maintain an immunodominant S1 epitope recognized by a protective monoclonal antibody. Moreover, certain of these mutants have been shown to protect mice at doses that exhibit minimal toxicity against challenge with virulent B. pertussis. While the majority of these results have been generated using PT mutants secreted by B. paraptussis, it is evident that equivalent products are obtained by genetic manipulation of B. pertussis itself. This disclosure, therefore, presents a number of detoxified immunogenic forms of pertussis toxin that would be candidates for inclusion in a novel pertussis vaccine, and a method for producing them in B. pertussis.

## EXAMPLES

Methods of molecular genetics, protein biochemistry and fermentation and hydridoma technology used but not explicitly described in this disclosure and these Examples are amply reported in the scientific literature and are well within the ability of those skilled in the art.

## Example I:

This Example illustrates the preparation and purification of PT.

Culture supernatants of B. pertussis. (strain 10536) were concentrated 20-50 times by ultrafiltration through a 10,000 or 20,000 molecular weight cut-off membrane using a Millipore Pellicon cassette system. The toxin was adsorbed from crude concentrates by passage through a fetuin-agarose affinity column equilibrated with 1 M potassium phosphate, 10 mM NaCl at pH 7.5. The volume of adsorbent was typically 1 ml per mg of toxin. The loaded column was washed with 100 mM potassium phosphate, 1 M NaCl at pH 7.5, then eluted with the same buffer containing 3 M potassium thiocyanate to desorb the toxin. Pooled fractions were dialyzed against 50 mM Tris-HCl, 200 mM NaCl containing 10% v/v glycerol at pH 8.0, to remove thiocyanate, then against 50 mM Tris-HCl, 200 mM NaCl containing 50% v/v glycerol at pH 8.0, to allow storage of the product at -200C. The yield as determined by ELISA was typically 90-95%. The purity as determined by SDS-PAGE was 90-95%, the major contaminant being FHA. For further purification the stored toxin was diluted five-fold with water and loaded onto a hydroxyapatite column of volume 1 ml per mg of toxin, that had been equilibrated with 10 mM potassium phosphate at pH 8.0. The column was washed with 30 mM potassium phosphate at pH 8.0 then eluted with 100 or 200 mM potassium phosphate to desorb the toxin. Pooled fractions were dialyzed against 100 mM potassium phosphate containing 50% v/v of glycerol at pH 8.0 and the final product stored at -200C. The yield was typically 90-95%, and the purity >99% as shown by SDS-PAGE.

## Example II:

This Example illustrates the preparation of PT subunit S1.

PT was adsorbed to fetuin-agarose as described in Example I, then the column was washed with CHAPS buffer (500 mM urea, 50 mM potassium phosphate, 100 mM NaCl and 1% w/v of CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate) at pH 7.5). The column was eluted with the same medium containing 500  $\mu$ M of adenosine triphosphate (ATP). The S1 subunit emerged as a sharp peak at the column volume. The pooled fractions were passed through a clean fetuin-agarose column equilibrated with CHAPS/ATP medium to remove residual B oligomer, then dialyzed against 100 mM potassium phosphate containing 50% v/v glycerol at pH 8.0 for storage at -200C. S1 was quantified by reverse-phase HPLC on a Vydac C4 column by comparison of the integrated peak area with that of a PT standard. The yield was typically only 20-25%, but the product was free of other subunits as demonstrated by both SDS-PAGE and reverse-phase HPLC.

## Example III:

This Example illustrates the photocrosslinking of NAD to the S1 subunit.

Reaction mixtures (100  $\mu$ l) containing 50  $\mu$ g/ml of S1, 10 mM dithiothreitol and 50  $\mu$ M NAD in CHAPS buffer were placed in the wells of a 96-well microtitre plate set in ice, preincubated for 30 min and then irradiated at 254 nm for periods up to 3 hr at a distance of 5 cm with a 9 W mercury lamp. Samples were then assayed for residual NAD glycohydrolase activity. The enzyme activity of S1 was completely abolished after irradiation for 2 hr, whereas the extent of photoinactivation was only 40% under the same conditions but in the absence of NAD. This result indicated that NAD-dependent photochemical events had occurred. To discover which part of the NAD molecule interacted with the protein and the extent of crosslinking, S1 was irradiated under identical conditions with [carbonyl- $^{14}$ C]NAD or [adenine- $^{14}$ C]NAD. Aliquots were removed at intervals up to 3 hr and treated with trichloroacetic acid (TCA) to 10% w/v. The precipitated protein was collected by filtration, washed with fresh 10% w/v TCA and counted in a scintillation counter. Results indicated that the radiolabel was incorporated from the nicotinamide moiety rather than from the adenine moiety, and that the extent of incorporation was 0.75 mol label per mol protein.

## Example IV:

This Example identifies the site of photocrosslinking on the S1 subunit.

Reaction mixtures (3 ml) containing 100  $\mu$ g/ml of S1, 10 mM dithiothreitol and 50  $\mu$ M [carbonyl- $^{14}$ C]-NAD in CHAPS buffer were placed in a Petri dish on ice to give a 1 mm layer, then irradiated at 254 nm for 2 hr with gentle magnetic stirring. The solution was deaerated with nitrogen, further reduced with dithiothreitol and S-alkylated with 4-vinylpyridine to prevent oxidation of thiol groups. The reaction mixture was dialyzed extensively against 10 mM acetic acid and the radiolabelled protein was collected after

precipitation with 20% w/v TCA.

The precipitated protein (1 mg) was redissolved in 2 M urea, 200 mM ammonium bicarbonate to 500 µg/ml and digested with 50 µg/ml trypsin for 20 hr at 37 °C. The mixture was acidified and fractionated on a 1x25cm Vydac C<sub>18</sub> reverse-phase HPLC column, using a linear gradient of 0-50% acetonitrile in 10 mM trifluoroacetic acid (TFA). Fractions were checked by scintillation counting, which revealed two major radioactive peptides, denoted A and B, accounting for 50% of the eluted radioactivity. The peptide pool was lyophilized, redissolved in 10 mM TFA, 6 M guanidinium chloride and separated on a Vydac C18 column using a 20-30% acetonitrile gradient in 10 mM TFA. Each peptide was further purified to homogeneity on the same column by applying an acetonitrile gradient in 20 mM ammonium acetate at pH 6.5, and the solutions evaporated to dryness. Their specific radioactivities were consistent with only one labelled site per molecule.

The two peptides were sequenced by automated Edman degradation. A portion of the sequenator effluent was diverted for monitoring of radioactivity. The results are shown in Figure 1. Up to cycle 15, the sequences proved to be identical and correspond unequivocally to residues 118-132 of mature S1. In both peptides radioactivity was associated with an unidentified amino acid released at cycle 12, corresponding to position 129 in mature S1. No radioactivity was detected at cycles beyond 15. Thus it was established that GLU<sup>129</sup> was the site of crosslinking, and is therefore likely to be an important component of the nicotinamide interaction site.

#### Example V:

This Example illustrates the preparation of *B. pertussis* chromosomal DNA.

Two litres of *B. pertussis* (strain 10536) grown in modified Stainer-Scholte medium as 16x125 ml aliquots using a 4 ml inoculum of saturated growth for each flask. This medium consists of L-proline 5g/L, NaCl 2.5 g/L, KH<sub>2</sub>PO<sub>4</sub> 0.5 g/L, KCl 0.2 g/L, MgCl<sub>2</sub>.6H<sub>2</sub>O 0.1 g/L, Tris 1.5 g/L, casamino acids 10 g/L, methyl-β-cyclodextrin 2 g/L, CaCl<sub>2</sub>.2H<sub>2</sub>O 0.02 g/L, mono-sodium glutamate 10g/L, L-cysteine 0.004%, FeSO<sub>4</sub>.7H<sub>2</sub>O 0.001%, niacin 0.004%, glutathione 0.015%, and ascorbic acid 0.04%, pH 7.6. Samples were grown in 500 ml flasks, on a shaker at 35-36 °C, 150 rpm for 16.5 hr to log phase. The cells were spun in 500 ml aliquots at 5000xg for 1 hr at 4 °C. Each aliquot was washed with 25 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5,) then resuspended in 20 ml TE and frozen at -70 °C. One pellet was resuspended in 90 ml TE and pronase added to 500 µg/ml. SDS was added to 1% and the sample incubated at 37 °C for 21.5 hr generating a clear lysate. The lysate was extracted with 1 volume of phenol saturated Tris-HCl at pH 7.5 at room temperature for 2 hr, with gentle agitation. The phases were separated by centrifugation at 2800xg for 15 min at 20 °C and the aqueous phase extracted similarly with 1 volume of 1:1 phenol:chloroform. The phases were separated by centrifugation at 2100xg for 10 min at 20 °C and the aqueous phase extracted with chloroform for 2 hr as described. The phases were separated by centrifugation at 1600xg for 5 min at 20 °C and the aqueous phase subjected to dialysis at 4 °C against 2 L of 1 M NaCl for 24 hr with one change of buffer, then against 2 L TE for 48 hr with one change of buffer.

#### Example VI:

This Example illustrates the generation of *B. pertussis* gene libraries.

##### 1) λ gIII EcoR I library

*B. pertussis* DNA (10 µg) was digested with EcoR I (10 units) in the presence of 100 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 100 µg/ml BSA, 1 µg/ml RNase A for various lengths of time in order to generate a set of partially digested DNA fragments. At each time point of 0.25, 0.5, 1, 2, 4 and 8 hrs, the sample was placed at 0 °C and EDTA added to 20 mM to stop the reaction. The samples were pooled and separated on a 10-40% sucrose gradient in TNE (20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1M NaCl) at 85,000xg for 20 hr at 20 °C. The gradient was fractionated from the top as 24 aliquots (0.5 ml) to which 1 ml aliquots of absolute ethanol were added to precipitate the DNA. The samples were incubated on dry ice for 30 min then centrifuged at 12,000xg for 5 min at 4 °C. The pellets were washed with 750 µl of 70% ethanol, incubated on dry ice for 5 min, centrifuged at 12,000xg for 5 min, then dried. Each pellet was resuspended in 23 µl of sterile water and 5 µl aliquots of every alternate fraction were submitted to agarose gel electrophoresis to determine the size of the fragments. Samples containing DNA ranging in size from approximately 0.5 kb to 9 kb were pooled. The pooled EcoR I-digested *B. pertussis* DNA (0.4 µg) was ligated with EcoR I-digested, dephosphorylated λ gIII DNA (0.5 µg) and was packaged into phage particles



using a commercial kit. The phage library was propagated in *E. coli* Y1090 cells and was titred at approximately  $10^{10}$  plaque-forming units(pfu)/ $\mu$ g of  $\lambda$  gtlI DNA. The library was amplified to  $4 \times 10^{10}$  pfu/ml for screening clones. The amplification was performed on plates by growing cells to saturation overnight in media containing 0.2% maltose, then adding  $10^4$  to  $10^5$  pfu of library per 0.6 ml of cells and allowing the  
 5 phage to adsorb to the cells for 15 min at  $37^\circ\text{C}$ . The sample was mixed with soft agar, plated, and incubated overnight at  $37^\circ\text{C}$ . The soft agar/cells/phage layer was scraped from the confluent plates which were washed with 4 ml SMG buffer (0.4 M NaCl, 10 mM  $\text{MgSO}_4$ , 50 mM Tris-HCl, pH 7.5, 0.01% gelatin). The wash and phage agar were combined, 100  $\mu$ l of chloroform added, and the mixture incubated at  $37^\circ\text{C}$  for 15 min with gentle agitation. The sample was centrifuged at  $4000 \times g$  at  $4^\circ\text{C}$  for 10 min twice to obtain a  
 10 clear supernatant. Chloroform was added to a final concentration of 0.3% and the library stored at  $4^\circ\text{C}$

## 2) $\lambda$ Charon 35 Sau3A I library

*B. pertussis* DNA (3x166  $\mu$ g) was digested with Sau3A I (3x220 units) in the presence of 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM  $\text{MgCl}_2$ , 100  $\mu$ g/ml BSA for 1 min, 2 min, or 3 min in order to generate very large fragments of DNA. After each reaction, EDTA was added to 20 mM and then 2.5 volumes of absolute ethanol added to precipitate the DNA as described above. The DNA was resuspended in TNE and separated on a 10-30% sucrose in gradient in TNE as described above. Fractions were taken as before and the DNA fragment sizes visualized by agarose gel electrophoresis.  $\lambda$  Charon 35 DNA (2x50  $\mu$ g) was ligated  
 20 to generate a circularized form before being digested with BamH I (2x20 units) in the presence of 150 mM NaCl, 6 mM Tris-HCl pH 7.9, 6 mM  $\text{MgCl}_2$ , 100  $\mu$ g/ml BSA to remove the stuffer fragments. The lambda arms were purified by pelleting through an 8-20% potassium acetate gradient at  $85,000 \times g$ , for 16 hr at  $32^\circ\text{C}$ . The Sau3A I digested DNA was ligated with the lambda arms at  $6^\circ\text{C}$  for 72 hr, then packaged into phage using a commercial kit. The phage library was propagated in *E. coli* LE392 cells and was titred at approximately  $1 \times 10^5$  pfu/ $\mu$ g of lambda arms. The library was amplified to  $1-2 \times 10^{10}$  pfu/ml for screening as described above.  
 25

## Example VII:

30 This Example illustrates the screening of the *B. pertussis* libraries.

### 1) $\lambda$ gtlI genomic library

A 30-base oligonucleotide probe was synthesized based on the nucleotide sequence of the gene encoding PT subunit S4. The DNA was purified from urea/acrylamide gels by uv-imaging and anion exchange chromatography on Whatman cellulose DE52. The sequence of the oligonucleotide was 5'-GTAG-CCATGAAGCCGTATGAAGTCACCCCG3', coding for amino acids 16-25 of the mature S4 protein. The oligonucleotide was 5' end-labelled in a reaction mix containing 10  $\mu$ g DNA, 25  $\mu$ Ci [8- $^{32}\text{P}$ ]ATP, 4 units polynucleotide kinase in the presence of 50 mM Tris-HCl, pH 9.5, 10 mM  $\text{MgCl}_2$ , 5 mM DTT, 5% glycerol  
 40 by incubation at  $37^\circ\text{C}$  for 15 min. ATP was added to 1.5 mM and the incubation continued for 1.75 hr at  $37^\circ\text{C}$ . 10  $\mu$ g of tRNA were added as carrier and the labelled DNA was separated from free ATP on a Sephadex G50 superfine column eluted with 0.1 M triethylammonium bicarbonate, pH 7.6. Peak fractions were pooled and lyophilized to dryness. The pellet was washed with sterile water, lyophilized then resuspended at approximately 0.1  $\mu$ g/ $\mu$ l.

45 Aliquots of the  $\lambda$  gtlI *B. pertussis* genomic library were plated on a Y1090 lawn on NZCYM plates containing 0.2% maltose. Plaque-lifts were made onto nitrocellulose filters which were sequentially treated with denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 1 min, neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl pH 8.0) for 5 min, and rinsed briefly in 2xSSPE (0.36 M NaCl, 20 mM sodium phosphate, pH 7.4, 2 mM EDTA) before being baked at  $80^\circ\text{C}$  under vacuum for 2 hr to fix the DNA. Nitrocellulose filters were  
 50 subsequently incubated in a prehybridization buffer comprising 5xSSC (0.75 M NaCl, 75 mM sodium citrate, pH 7.5), 5x Denhardt's mixture (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% BSA), 0.1% SDS, 100  $\mu$ g/ml herring sperm DNA for 2 hr at  $45^\circ\text{C}$ . The prehybridization buffer was removed and fresh buffer containing  $10^7$  cpm of [ $^{32}\text{P}$ ]-labelled oligonucleotide probe was added. Hybridization was carried out at  $45^\circ\text{C}$  for 16 hr. The radioactive solution was removed and the filters rinsed briefly twice at room  
 55 temperature with 5xSSC, 0.1% SDS to remove unbound probe. The filters were further washed twice with 5xSSC, 0.1% SDS for 1 hr at  $50^\circ\text{C}$  then air-dried and subjected to autoradiography.

The plaque-containing plates were aligned with their autoradiograms and putative positive plaques were subjected to another two rounds of purification on plates. One clone ( $\lambda$  gtlI-15-4-1) was chosen for detailed

examination by Southern blot analysis.

## 2) $\lambda$ Charon 35 genomic library

- 5 Aliquots of the  $\lambda$  Charon 35 B. pertussis genomic library were plated on an LE392 lawn on NZCYM plates containing 0.2% maltose. The plaque-lift, hybridization and washing protocols were performed as described. Positive plaques were purified twice more on plates and several clones,  $\lambda$  Ch 35 111, 121, 411, 421 and 431, were examined by Southern blot analysis.

## 10 Example VIII:

This Example illustrates the analysis of the genomic clones.

### 1) Preparation of phage DNA

15

One litre (2x500 ml) of phage culture was prepared. LE392 or Y1090 cells were grown overnight in medium containing 0.2% maltose. Cells ( $10^{10}$ ) were spun down at 4400xg for 5 min at 4°C and the pellet resuspended in 1 ml SMG buffer. Phage stock ( $1.2 \times 10^8$  pfu) was added to the mixture and incubated at 37°C for 15 min to absorb the phage to the cells. The phage/cell mixture was inoculated into 500ml of  
20 medium and the culture shaken vigorously at 37°C until lysis began (4-4.5 hr). Chloroform (10ml) was added and shaking continued at 37°C for an additional 15 min to complete the lysis. The sample was cooled to room temperature and DNase I and DNase-free RNase A (1  $\mu$ g/ml each) were added for 30 min at room temperature. The cell debris was pelleted at 3500xg for 20 min, then 29.2 g NaCl and 50 g polyethylene glycol (PEG 6000) were added to 500 ml of supernatant. The sample was gently agitated at  
25 room temperature to dissolve the solids, then incubated at 0°C for 1-2 hr to precipitate the phage. The phage were harvested by centrifuging at 4400xg at 4°C for 20 min and were resuspended in 8 ml TM buffer (50 mM Tris-HCl, pH 7.5, 10 mM  $MgSO_4$ ). Extraction with 8 ml chloroform to remove the PEG gave a clear supernatant which was applied to a step gradient of 5% and 40% glycerol in TM buffer and centrifuged at 154,000xg at 4°C for 1hr. The supernatant was discarded leaving a phage pellet which was  
30 resuspended in 0.5 ml TM buffer. DNase I was added to 5  $\mu$ g/ml and RNase A to 50  $\mu$ g/ml and the sample incubated at 37°C for 30 min. EDTA was added to 20 mM, pronase to 0.5 mg/ml, SDS to 0.5%, and the sample further incubated at 37°C for 1hr. The sample was gently extracted once each with phenol, phenol:chloroform 1:1, and chloroform and the phage DNA precipitated with ethanol.

### 35 2) Results

Clone 15-4-1 which was derived from the EcoR I gtlI library, was found by Southern blot analysis to contain the 4.6 kb EcoR I fragment encoding the entire TOX gene plus small 5'- and 3'-flanking regions.

The  $\lambda$  Charon 35 clones were found to be closely related. Some clones contained the entire TOX  
40 operon plus flanking regions in either orientation, and others did not include the entire TOX region.

The maps of clones 15-4-1, Ch 111, Ch 121/411, Ch 431 and Ch 421 are shown in Figure 2.

## Example IX:

45 This Example illustrates the construction of pUC-based plasmids containing the pertussis toxin operon (TOX) or portions thereof.

Phage DNA from the  $\lambda$  gtlI clone 15-4-1 was prepared as described and digested with restriction endonuclease EcoR I using standard methods. The DNA was purified by gel electrophoresis in low-melting-point agarose. The 4.6 kb band was identified by uv-illumination of the ethidium bromide stained gel and  
50 excised. The DNA was extracted by a freeze-thaw technique employing 0.3 M sodium acetate, pH 7.0 and was precipitated with ethanol. DNA from pUC8:2, a derivative of pUC8 containing two extra restriction sites for Bgl II and Xba I in its multiple cloning site, was digested with EcoR I. The linearized DNA was dephosphorylated by standard methods using calf alkaline phosphatase (CAP), phenol extracted and precipitated with ethanol.

55 The pUC8:2-vector DNA and 15-4-1-derived-TOX DNA were ligated in a standard reaction and the ligation mixture used to transform competent JM109 cells according to standard procedures. The resulting colonies were analysed by a rapid DNA screening technique and two clones were chosen for large-scale preparation of plasmid DNA. These clones, J-169-1 and J-169-2, differed only in the orientation of the TOX

insert. The construction of these clones is illustrated in Figure 3.

#### Example X:

5 This Example illustrates the sequencing of the TOX operon. 1) Clones used

The clone J-169-1 was used as the source for all sequencing clones. The TOX operon was divided into five approximately equal DNA segments and was subcloned into M13mp18, M13mp19 or pUC8:2 as illustrated in Figures 4a, b, c, d and e.

10 2) Preparation of samples

M13 clones were maintained in JM101 and DNA for sequencing was prepared from single plaques on homogeneous plates. A saturated JM101 culture was diluted 1:50 with fresh medium and infected with a single plaque. The culture was grown with vigorous shaking at 37°C for 6 hr. The cells were removed by  
15 centrifugation and the supernatant treated with 1/4 volume of 20% PEG 6000, 2.5 M NaCl to precipitate phage. The suspension was centrifuged and the phage pellet was resuspended in TE, then extracted gently twice each with phenol, phenol:chloroform (1:1) and chloroform. The phage DNA was precipitated with sodium acetate and ethanol, washed with 70% ethanol and dried. The DNA was resuspended in sterile water to a concentration of about 1 µg/ml for sequencing.

20 Sequencing primers of approximately 17-20 bases were synthesized on an ABI 380A DNA synthesizer using phosphoroamidite chemistry and were purified as described above.

3) Sequencing

25 The dideoxy chain termination method of Sanger was used for all sequencing reactions, employing either Klenow polymerase or Sequenase T7 enzyme.

4) Results

30 The entire TOX operon, as previously defined, was sequenced and the result compared with published sequences. There was excellent agreement with the TOX sequence of strain BP 165 reported by Nicosia et al., except for four base differences. The T at position 315 in the 5'-flanking region is unique to B. pertussis strain 10536. The three other substitutions are in the S1-coding region at positions 710, 1200 and 1202 resulting in two unique amino acids, GLU34 and VAL198. The nucleotide sequence and derived amino acid  
35 sequence are shown in Figure 5.

#### Example XI:

This Example illustrates mutagenesis of the TOX gene.

40

1) Clones used

For mutations in the S1 gene, clone S-2403 (M13mp18/S1) was used and for the mutations in the S3 gene, clone S-2664-5-6, (M13mp18/S3(c)) was used. These clones are represented in Figure 4.

45

2) Mutagenesis protocol

Single-stranded DNA was prepared from phage stocks derived from single plaques on homogeneous plates as described previously. Mutagenic primers of appropriate sequence and length were synthesized on  
50 an ABI 380A DNA synthesizer.

Commercial kits based on the phosphorothioate procedure developed by Eckstein were used for in vitro mutagenesis. Briefly, the mutagenic oligonucleotide was annealed to the single-stranded (wild-type) template and polymerization carried out using as substrates a phosphorothioate dCTP analogue and natural dATP, dGTP and dTTP. The double-stranded DNA was nicked with Nci I and the native strand digested  
55 with exonuclease III beyond the point of the mutation. The complementary strand was protected from Nci I-nicking by the phosphorothioate groups. The complementary strand then served as a template in a second round of polymerization, to yield double-stranded DNA with the mutation in both strands. This DNA was amplified in E. coli, and the mutation confirmed by sequencing.

Thirty-five primary mutations were generated and an additional 14 were derived by constructing crosses among these. The mutation efficiency varied with the change desired. From one to six base changes and deletions of up to 15 consecutive bases were accomplished. The resulting amino acid changes are summarized in Table 1a.

#### Example XII:

This Example describes the construction of plasmids for expression of mutated TOX genes in B. parapertussis and characterization of the PT analogues produced.

##### 1) Replicating plasmids

Replicative-form DNA from M13 clones was used to reconstruct the TOX operon containing the desired mutation in pRK404. pRK404 is a derivative of pRK290, a conjugating plasmid of the pRK2 family, incompatibility group P-1. It is 10.6 kb in size, carries a tetracycline resistance (Tet<sup>R</sup>) gene, and has a multiple cloning site from pUC8. The construction schemes for reintegrating S1 and S3 primary mutations into the operon are shown in Figure 6 and the resulting clones are indicated in Table 1a. Crossed mutations in S1 were generated using internal restriction sites, especially the unique Sal I site. A general scheme for crossed mutations in S1 is also shown in Figure 6 and the resulting clones are indicated in Table 1a.

##### 2) Suicide plasmids

A conjugative but non-replicative plasmid was developed for random integration of TOX or mutated TOX into the chromosome of Bordatella species. Figure 7 demonstrates the construction of these clones.

Plasmids of the types described in (1) and (2) above were introduced into B. pertussis by conjugation. The resulting strains were grown in shake-flasks or in a fermentor, and the culture supernatants were assayed as follows for concentration of toxin analogue by ELISA. Microtitre plates were coated with fetuin (2 µg/ml) in 0.05 M potassium carbonate, pH 9.6 at 4 °C overnight in a humid environment. The plates were then washed twice with Delbecco's PBS containing 0.1% w/v Tween-20 and dried. Sample supernatants or wild-type PT were serially diluted and added to the wells, and the plates incubated for 30 min at room temperature then washed. Bound PT was detected using peroxidase-conjugated affinity-purified rabbit anti-PT antibodies.

Residual toxicity was measured by the CHO cell clustering assay, to determine the toxicity relative to native PT. Certain PT mutants were purified as described for native PT in Example I, and assayed for ADP-ribosyltransferase activity. These data are summarized in Table 1b. Expression of the S1 epitope recognized by MAb PS21 was assessed by a modified indirect ELISA on culture supernatants. Fetuin-bound PT analogues were reacted with PS21 as the first antibody and visualized with an enzyme-conjugated affinity-purified goat anti-mouse IgG as the second antibody. The presence or absence of the S1 epitope recognized by MAb PS21 is indicated in Table 1b.

#### Example XIII:

This Example illustrates the construction of plasmids for deletion and replacement of the endogenous B. pertussis TOX operon.

##### 1) Plasmids containing TOX flanking regions

###### a) 5'-flanking region

The Ch 421 DNA was first digested with Bgl II and an 11 kb fragment was purified by agarose gel electrophoresis. The Bgl II fragment was digested with Xma I and the 5 kb band subcloned into pUC8:2 previously restricted with Xma I and dephosphorylated. JM109 cells were transformed with the ligation mixture to give colonies which were analysed by a rapid DNA screening method. The clone J-183-9 was found to contain approximately 2.9 kb of the 5'-flanking region, the TOX promoter and the genes for subunits S1 and S2. Figure 8a shows the derivation of clone J-183-9.

###### b) 3'-flanking region

The Ch 111 DNA was digested with Sal I and an approximately 8 kb fragment of B. pertussis DNA was gel-purified. This DNA fragment was inserted into pUC8:2 previously digested with Sal I and dephosphorylated. JM109 transformants were screened and the clone J-219-111-3 was identified as

containing part of the S1 gene, all of the remaining structural genes, and about 3.9 kb of the 3' flanking region. Figure 8b shows the construction of this clone.

c) TOX gene with 5'- and 3'-flanking regions.

Clone J-183-9 was digested with Xba I and the approximately 7 kb fragment containing pUC8:2, the 5'-flanking region and the promoter region of the S1 gene was gel-purified and dephosphorylated. J-219-111-3 DNA was digested with Xba I and the approximately 8 kb fragment containing the structural genes for subunits S2 to S5 and the 3'-flanking regions was gel-purified. These DNA fragments were ligated and the JM109 transformants were screened to give clone J-229-17. This clone contains about 2.9 kb of the 5'-flanking sequence, the entire TOX operon, and about 4 kb of the 3'-flanking sequence. Its construction is illustrated in Figure 8c.

2) TOX-deleting plasmids

Plasmid S-2832-5 contains the Tet<sup>R</sup> gene from plasmid pRK404 and its construction is shown in Figure 9. The Tet<sup>R</sup> gene was cloned as an EcoR I/BamH I restriction fragment into plasmid pN01523 to generate pGZ62. Plasmid pGZ63 contains the 5'- and 3'-flanking regions without any intervening DNA. The S12-Tet<sup>R</sup> gene-sandwich from pGZ62 was cloned between the flanking regions of pGZ63 to produce plasmid pGZ65. The construction of these plasmids is summarized in Figure 8d.

3) TOX-reintegrating plasmids

To express mutated TOX genes in TOX<sup>-</sup> strains of B. pertussis conjugative suicide plasmids of the type shown in Figure 10 were constructed. They contain the TOX gene, extensive 5'- and 3'-flanking sequences and have a Tet<sup>R</sup> gene for selection cloned downstream from the TOX coding regions.

Example XIV:

This Example illustrates the deletion of the TOX gene from the B. pertussis chromosome and the reintegration of in vitro-mutated TOX genes.

1). Transformation of B. pertussis

Strains of B. pertussis were transformed by electroporation. Cells were grown in 100 ml of modified Stainer-Scholte medium to a density of about 109 cells/ml, harvested in a clinical centrifuge (4000xg for 15 min at 20 °C), washed in 25 ml of electroporation buffer (0.3 M sucrose, 1 mM MgCl<sub>2</sub>, 7 mM potassium phosphate, pH 7.2) and resuspended in 10 ml of the same. Plasmid DNA was added to 500 µl of the cell suspension and the mixture incubated on ice for 10 min. The cells were subjected to a single 25 kV/cm, 40 µs exponential decay voltage pulse with a BTX Transfector 100, using a cuvette electrode with a 0.8 mm gap. Three ml of medium were added and the cells incubated with shaking at 37 °C for 60 min. The cells were harvested by centrifugation at 12,000xg for 2 min, resuspended in 100 µl of medium, spread onto a Bordst-Gengou plate with antibiotic selection and incubated for 2-5 days at 37 °C.

a) Deletion and replacement of the TOX operon

B. pertussis str29 is a spontaneous rpsL streptomycin resistant derivative of B. pertussis 10536. Plasmid pGZ65 contains a gene cartridge consisting of the pRK404 Tet<sup>R</sup> gene and the E. coli S12 gene cloned between the 5'- and 3'-flanking sequences of the TOX operon. This plasmid was linearized with Hind III and used to transform B. pertussis str29 to Tet<sup>R</sup>, Str<sup>S</sup> resulting in the deletion of the TOX operon by homologous recombination. This TOX-deleted strain was termed 29-8. To excise the S12-Tet<sup>R</sup> gene cartridge, strain 29-8 was subsequently transformed with linear pGZ63 plasmid DNA. Plasmid pGZ63 consists of the TOX 5'- and 3'-flanking sequences but contains no intervening DNA. Transformation with this plasmid resulted in the generation of B. pertussis 29-9 which is a streptomycin-resistant, TOX-deleted strain but contains no heterologous DNA inserted at the TOX locus. This strain was used as the host for expression of in vitro mutated TOX genes. Plasmids of the type shown in Figure 10 contain a gene cartridge consisting of a mutated TOX gene and a Tet<sup>R</sup> gene. This gene cartridge was recombined into the B. pertussis 29-9 chromosome following introduction of the plasmid into the strain by conjugation or transformation. Expression of the TOX gene, toxicity of the PT analogues and maintenance of the S1 epitope recognised by MAbs PS21 were determined as described before. The recombinant B. pertussis strains constructed and the properties of the secreted PT analogues are shown in Table 5.

## Example XV:

This Example describes the in vivo testing of PT mutants in mice.

PT mutants were purified from culture supernatants and recombinant B. paraptussis strains as indicated in Example I. These proteins were injected into mice at three different doses to test the following characteristics, according to standard procedures: acute toxicity, histamine sensitization activity and potency in the mouse intracerebral challenge test. The results are presented in Table 2.

To test their immunogenicity, PT analogues were injected into female BALB/C mice, 9 to 11 weeks old, at doses of 2.0, 0.5 and 0.125  $\mu$ g. Mice were pre-bled and immunized on day 0. On day 23 the mice were bled again and boosted with the same immunogen, and on day 37 the mice were bled again. Blood samples (0.4 - 0.5 ml/mouse) were collected by orbital sinus bleeding and the resulting sera stored at -20°C to await testing. Sera were assayed for their ability to neutralize PT-induced CHO cell clustering (Table 3), and for specific antibody responses in antigen-coat, indirect ELISA (Table 4). As may be seen from Tables 3 and 4, PT analogues are capable of inducing neutralizing antibodies and pool anti-PT, anti-S1 and anti-B oligomer responses.

SUMMARY OF DISCLOSURE

In summary of this disclosure, the present invention provides a novel method of detoxifying pertussis by identification of specific functional sites of pertussis toxin and production of recombinant holotoxins by site-directed mutagenesis of the toxin gene. The resulting toxin analogues are detoxified, retain an immunodominant S1 epitope, are immunogenic and are protective against the disease pertussis. Modifications are possible within the scope of this invention.

TABLE 1a

## Summary of Mutations introduced into Pertussis Toxin

5	Mutation Number	Mutation	Clone No.
	1.	ARG <sup>9</sup> -> Δ <sup>9</sup>	S-2679-1-11
	2.	" -> GLU <sup>9</sup>	S-2815-1-3
10	3.	" -> LYS <sup>9</sup>	S-2953-21
	4.	" -> HIS <sup>9</sup>	S-3046-4
	5.	ARG <sup>13</sup> -> Δ <sup>13</sup>	S-2679-2-1
	6.	" -> GLU <sup>13</sup>	S-2779-2-1
	7.	ARG <sup>9</sup> -ARG <sup>13</sup> -> Δ <sup>9-13</sup>	S-2829-2-19
15	8.	ARG <sup>9</sup> ARG <sup>13</sup> -> GLU <sup>9</sup> GLU <sup>13</sup>	S-2779-3-2
	9.	ARG <sup>58</sup> -> GLU <sup>58</sup>	J-444-2-2
	10.	ARG <sup>57</sup> ARG <sup>58</sup> -> Δ <sup>57</sup> Δ <sup>58</sup>	J-482-11
	11.	TYR <sup>26</sup> -> ALA <sup>26</sup>	S-3123-2
	12.	" -> CYS <sup>26</sup>	S-3140-22
20	13.	CYS <sup>41</sup> -> ALA <sup>41</sup>	S-2515-5-10
	14.	" -> SER <sup>41</sup>	S-3124-6
	15.	CYS <sup>201</sup> -> ALA <sup>201</sup>	S-2679-3-4
	16.	GLU <sup>129</sup> -> Δ <sup>129</sup>	S-2589-6
	17.	" -> GLY <sup>129</sup>	S-2515-3-6
25	18.	" -> GLN <sup>129</sup>	S-2515-1-2
	19.	" -> ASP <sup>129</sup>	S-2515-2-4
	20.	" -> ASN <sup>129</sup>	S-2852-1-18
	21.	" -> LYS <sup>129</sup>	S-2515-4-11
	22.	" -> ARG <sup>129</sup>	M-32-2-4
30	23.	" -> HIS <sup>129</sup>	S-2937-1-2
	24.	" -> PRO <sup>129</sup>	S-2959-2-29
	25.	" -> CYS <sup>129</sup>	J-478-5
	26.	" -> GLY <sup>129</sup> II	J-418-1
	27.	" -> GLN <sup>129</sup> II	J-412-9
35	28.	TYR <sup>130</sup> -> Δ <sup>130</sup>	S-2852-2-1
	29.	" -> PHE <sup>130</sup>	S-2836-15
	30.	GLU <sup>129</sup> TYR <sup>130</sup> -> GLY <sup>129</sup> ALA <sup>130</sup>	S-2679-4-3
	31.	GLU <sup>129</sup> TYR <sup>130</sup> -> GLN <sup>129</sup> ALA <sup>130</sup>	M-38-1
40	32.	GLU <sup>129</sup> TYR <sup>130</sup> -> GLY <sup>129</sup> PHE <sup>130</sup>	J-444-1-6

45

50

55

TABLE 1a (c n't)

Mutation Number	Mutation	Clone No.
33.	(S3) LYS <sup>10</sup> -> GLN <sup>10</sup>	S-2995-1-2
34.	(S3) TYR <sup>92</sup> LYS <sup>93</sup> -> ASN <sup>92</sup> ARG <sup>93</sup>	S-2995-2-1
35.	(S3) LYS <sup>105</sup> -> ASN <sup>05</sup>	S-2995-3-1
36.	CYS <sup>41</sup> CYS <sup>201</sup> -> ALA <sup>41</sup> ALA <sup>201</sup>	S-2818-1
37.	CYS <sup>41</sup> GLU <sup>129</sup> -> ALA <sup>41</sup> GLY <sup>129</sup>	S-2549-2
38.	ARG <sup>9</sup> GLU <sup>129</sup> -> GLU <sup>9</sup> GLY <sup>129</sup> II	S-2966-1-5
39.	ARG <sup>9</sup> GLU <sup>129</sup> -> GLU <sup>9</sup> GLN <sup>129</sup> II	S-2967-1-1
40.	ARG <sup>9</sup> GLU <sup>129</sup> -> GLU <sup>9</sup> ARG <sup>129</sup>	M-45-1
41.	ARG <sup>9</sup> GLU <sup>129</sup> TYR <sup>130</sup> -> GLU <sup>9</sup> GLY <sup>129</sup> ALA <sup>130</sup>	S-2956-1
42.	ARG <sup>13</sup> GLU <sup>129</sup> -> GLU <sup>13</sup> GLY <sup>129</sup> II	S-2966-2-13
43.	ARG <sup>13</sup> GLU <sup>129</sup> -> GLU <sup>13</sup> GLN <sup>129</sup> II	S-2967-2-17
44.	ARG <sup>13</sup> GLU <sup>129</sup> TYR <sup>130</sup> -> GLU <sup>13</sup> GLY <sup>129</sup> ALA <sup>130</sup>	S-2961-1
45.	ARG <sup>9</sup> GLU <sup>129</sup> -> Δ <sup>9</sup> GLN <sup>129</sup>	S-2730-1-1
46.	ARG <sup>9</sup> GLU <sup>129</sup> TYR <sup>130</sup> -> Δ <sup>9</sup> GLY <sup>129</sup> ALA <sup>130</sup>	S-2730-3-2
47.	ARG <sup>13</sup> GLU <sup>129</sup> -> Δ <sup>13</sup> GLN <sup>129</sup>	S-2730-2-1
48.	ARG <sup>13</sup> GLU <sup>129</sup> TYR <sup>130</sup> -> Δ <sup>13</sup> GLY <sup>129</sup> ALA <sup>130</sup>	S-2730-4-1
49.	GLU <sup>129</sup> -> GLY <sup>129</sup>	S-3050-1
	(S3) TYR <sup>92</sup> LYS <sup>93</sup> (S3) ASN <sup>92</sup> ARG <sup>93</sup>	
50.	Wild Type	S-2505-4-5

Amino acid numbering corresponds to positions in the native subunits (Figure 5)

All mutations are in subunit S1 unless specified as being in S3 (S3)

II denotes use of an alternative codon

Δ denotes deleted residue(s)

Wild type refers to PT expressed from the unmutated TOX operon in B. parapertussis.



TABLE 1b

In vitro characterization of pertussis toxin analogues  
obtained from recombinant B. parapertussis.

5	Mutation Number	Residual T x i l y	ADPR Activity	SI Epitope
	1.	0.2	ND	-
	2.	0.1	0.2	+/-
10	3.	0.1	ND	++++
	4.	0.2	0.1	+++
	5.	0.3	ND	-
	6.	5.0	ND	++++
15	7.	0.4	0.1	-
	8.	0.1	0.9	-
	9.	0.7	0.6	+++
	10.	0.4	ND	-
20	11.	0.5	ND	+
	12.	6.0	ND	ND
	13.	0.3	0.4	-
25	14.	1.4	ND	ND
	15.	0.2	0.1	-
	16.	0.1	ND	++
	17.	0.1	0.3	++++
	18.	0.02	0.1	+/-
30	19.	0.7	2.5	++
	20.	0.1	0.3	++
	21.	0.3	0.2	-
	22.	0.1	ND	-
	23.	0.2	ND	-
	24.	0.2	ND	+
35	25.	0.4	ND	-
	26.	0.1	0.3	++++
	27.	0.02	0.1	+/-
	28.	0.2	0.1	-
40	29.	12.0	ND	++++
	30.	0.2	0.6	-
	31.	0.4	ND	-
	32	1.0	ND	++++

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TABLE 1b (c n't)

Mutation Number	Residual T xicity	ADPR activity	S1 Epitope
33.	100	ND	+++++
34.	50	100	++++
35.	20	ND	++++
36.	0.2	0.1	-
37.	0.1	0.1	-
38.	0.1	0.1	-
39.	0.1	ND	-
40.	0.1	ND	-
41.	0.2	ND	-
42.	0.5	ND	-
43.	3.0	ND	-
44.	0.3	ND	-
45.	0.4	ND	-
46.	0.2	0.1	-
47.	0.5	ND	-
48.	0.4	0.3	-
49.	0.2	0.1	++++
50.	100	100	+++++

Residual toxicity is the ratio of the apparent PT concentration determined by the CHO cell clustering assay to the actual concentration of PT mutant determined by ELISA expressed as a percentage.

ADPR activity is the extent of ADP-ribosylation of bovine transducin catalysed by a PT analogue, relative to that catalysed by an equal concentration of wild-type PT, expressed as a percentage.

S1 epitope refers to the expression of an immunodominant S1 epitope recognized by a specific monoclonal antibody PS21, as compared with the wild-type PT (++++).

ND denotes not determined.

TABLE 2

Biological Activity of PT mutants in mice			
Analogue	Acute Toxicity LD <sub>50</sub> (ug)	HS Activity LD <sub>50</sub> (ug)	M.P.T. ED <sub>50</sub> (ug)
Native	~2	0.2	>2
GLY <sup>129</sup>	>5	~3	~ 2
GLN <sup>129</sup>	>16	>3	16
ASN <sup>129</sup>	>5	~3	1.5
GLU <sup>58</sup>	>5	1.5	8.5
GLY <sup>129</sup> (S3)ASN <sup>92</sup> ARG <sup>93</sup>	>20	7	2
(S3)ASN <sup>92</sup> ARG <sup>93</sup>	3	0.4	>2
<p>HS Activity denotes histamine sensitizing activity.</p> <p>M.P.T. denotes mouse intracerebral challenge protection test.</p> <p>LD<sub>50</sub> is the dose resulting in death of 50% of the test animals.</p> <p>ED<sub>50</sub> is the dose resulting in protection of 50% of the test animals.</p> <p>Native denotes PT from <u>B. pertussis</u> 10536.</p>			

TABLE 3

Neutralizing effect of immune sera on PT-induced CHO cell clustering			
Analogue Dose (ug)	Pre-bleed	Post-1 bleed	Post-2 bleed
GLY <sup>129</sup>			
2.0	<2	<2	256
0.5	<2	<2	128
0.125	<2	<2	64
GLN <sup>129</sup>			
2.0	<2	<2	128
0.5	<2	<2	256
0.125	<2	<2	128
ASN <sup>129</sup>			
2.0	<2	<2	512
0.5	<2	<2	128
0.125	<2	<2	256
Saline	<2	<2	<2
<p>Mice were pre-bled and immunized on day 0. On day 23 they were bled again (post-1 bleed) and boosted. Final sera were obtained on day 37 (post-2 bleed).</p> <p>The neutralizing ability of the sera is expressed as the maximum dilution at which CHO cell clustering was inhibited.</p>			

TABLE 4

Specific antibody titres of immune sera									
Analoge Dose (ug)	Pre-bleed			Post-1 bleed			Post-2 bleed		
	PT	S1	B	PT	S1	B	PT	S1	B
GLY <sup>129</sup>									
2.0	NR	NR	NR	63	2	50	500	80	200
0.5	NR	NR	NR	13	1	8	160	32	56
0.125	NR	NR	NR	10	0.5	8	200	32	80
GLN <sup>129</sup>									
2.0	NR	NR	NR	22	0.7	20	200	40	125
0.5	NR	NR	NR	8	0.5	6	200	40	100
0.125	NR	NR	NR	5	<0.5	2	125	20	50
ASN <sup>129</sup>									
2.0	NR	NR	NR	40	1	40	500	140	280
0.5	NR	NR	NR	7	<0.5	3	316	22	80
0.125	NR	NR	NR	7	<0.5	4	180	63	125
Saline	NR	NR	NR	NR	NR	NR	NR	NR	NR
Immunization and bleeding were performed as described in Table 3. Antigens used were PT holotoxin, isolated S1 subunit and isolated B oligomer. The units are the dilution factor divided by 1000 giving an ELISA absorbance value equal to twice the background. NR denotes not reactive with antigen.									

TABLE 5

In vitro characterization of pertussis toxin analogues from recombinant <i>B. pertussis</i>				
Mutation Number	Clone	Residual Toxicity	ADPR Activity	S1 Epitope
9	S-3036-2	0.2	0.3	+++
10	S-3122-3-1	0.1	ND	ND
17	S-2962-1-2	0.2	ND	ND
18	S-2962-2-1	0.1	ND	ND
34	S-3122-3-1	50	ND	+++++
49	S-3122-2-3	0.1	ND	+++
50	S-3006-3	100	100	+++++
All terms are as defined in Tables 1a and 1b. ND denotes not determined.				

## Claims

1. A mutant pertussis holotoxin obtained by expression in a *Bordetella* organism of a TOX operon encoding the holotoxin which has been mutated by site-directed mutagenesis of at least one codon encoding at least one functional amino acid within native pertussis holotoxin to effect removal or replacement of said at least one functional amino acid and to genetically detoxify said holotoxin to a residual toxicity of 1% or less while retaining immunoprotective properties.

2. A mutant holotoxin as claimed in Claim 1, characterised in that said site directed mutagenesis modifies the A portion (S1 subunit) of the holotoxin, the B portion of the holotoxin or both the A and B portions of the holotoxin.
- 5 3. A mutant holotoxin as claimed in Claim 1 or Claim 2, characterised in that a single amino acid in the native pertussis holotoxin is removed or replaced.
4. A mutant holotoxin as Claimed in Claim 3, characterised in that the single amino acid is (S1) GLU<sup>129</sup> and is removed or is replaced by (S1) GLY<sup>129</sup>, or is (S1) ARG<sup>9</sup> and is replaced by (S1) LYS<sup>9</sup>, or is (S1) ARG<sup>58</sup> and is replaced by (S1) GLU<sup>58</sup>.
- 10 10. A mutant holotoxin as claimed in Claim 1 or Claim 2, characterised in that multiple amino acids in the native pertussis holotoxin are removed or replaced.
- 15 6. A mutant holotoxin as claimed in Claim 5, characterised in that the multiple amino acids are (S1) GLU<sup>129</sup> TYR<sup>130</sup> and are replaced by (S1) GLY<sup>129</sup> PHE<sup>130</sup>, or are (S1) GLU<sup>129</sup>/(S3) TYR<sup>92</sup>LYS<sup>93</sup> and are replaced by (S1) GLY<sup>129</sup>(S3)ASN<sup>92</sup>ARG<sup>93</sup>.
- 20 7. A mutant holotoxin as claimed in any one of Claims 1 to 6, characterised in that (S1) ARG<sup>9</sup>, ARG<sup>13</sup>, TRP<sup>26</sup>, ARG<sup>58</sup> and/or GLU<sup>129</sup> is deleted or replaced.
8. A mutant holotoxin as claimed in any one of Claims 1 to 6, characterised in that (S1) ARG<sup>9</sup>, ARG<sup>13</sup> and/or GLU<sup>129</sup> is deleted or replaced.
- 25 9. A mutant holotoxin as claimed in any one of Claims 1 to 8, characterised by a residual toxicity less than about 0.5% of the toxicity of the native toxin.
- 30 10. A mutant holotoxin as claimed in any one of Claims 1 to 9, characterised in that the mutant possesses a decreased histamine sensitisation activity.
11. A vaccine against whooping cough, characterised by containing a mutant holotoxin as claimed in any one of Claims 1 to 10, or a toxoid thereof, and a physiologically-acceptable carrier therefor.
- 35 12. A conjugate vaccine, characterised by an active conjugate comprising a mutant holotoxin as claimed in any one of Claims 1 to 10, conjugated to a hapten, polysaccharide or polypeptide to elicit an immune response to an antigenic determinant of the hapten, polysaccharide or polypeptide.
- 40 13. A method for the production of an immunoprotective genetically-detoxified mutant of pertussis holotoxin, characterised by the steps of (a) identifying at least one amino acid residue of the toxin which alone or together confer toxicity to the holotoxin; (b) effecting site-directed mutagenesis to remove or replace a nucleotide sequence coding for said at least one amino acid residue and to produce a mutated TOX operon; (c) introducing the mutated tox operon into a Tox<sup>-</sup> Bordetella organism; and (d) growing the transformed organism to produce genetically-detoxified holotoxin having a residual toxicity of 1% or less while retaining immunoprotective properties in the absence of wild type toxin.
- 45 14. A method as claimed in Claim 13, characterised in that a single amino acid in the native pertussis holotoxin is removed or replaced.
- 50 15. A method as claimed in Claim 14, characterised in that said single amino acid is (S1) ARG<sup>9</sup>, (S1) GLU<sup>129</sup> or (S1) ARG<sup>58</sup> and is replaced by (S1) LYS<sup>9</sup>, (S1) GLY<sup>129</sup> or (S1) GLU<sup>58</sup> respectively.
16. A method as claimed in Claim 13, characterised in that multiple amino acids in the native pertussis holotoxin are removed or replaced.
- 55 17. A method as claimed in Claim 16, characterised in that said multiple amino acids are (S1) GLU<sup>129</sup> TYR<sup>130</sup> or (S1) GLU<sup>129</sup>/(S3) TYR<sup>92</sup> LYS<sup>93</sup> and are replaced by (S1) GLY<sup>129</sup> PHE<sup>130</sup> and GLY<sup>129</sup>/(S3) ASN<sup>92</sup> ARG<sup>93</sup> respectively.

18. A method as claimed in any one of claims 13 to 17, characterised in that (S1) ARG<sup>9</sup>, ARG<sup>13</sup>, TRP<sup>26</sup>, ARG<sup>58</sup> and/or GLU<sup>129</sup> is deleted or replaced.
19. A method as claimed in any one of Claims 13 to 17, characterised in that (S1) ARG<sup>9</sup>, ARG<sup>13</sup> and/or GLU<sup>129</sup> is deleted or replaced.
20. A method as claimed in any one of Claims 13 to 19, characterised in that the introduction step in effected by conjugation or by electroporation.
21. A TOX operon encoding a mutant pertussis holotoxin which has been mutated by site-directed mutagenesis of at least one codon encoding at least one functional amino acid within native pertussis holotoxin to effect removal or replacement of said at least one functional amino acid to genetically detoxify said holotoxin to a residual toxicity of 1% or less while retaining immunoprotective properties.
22. A TOX operon as claimed in claim 21 wherein said site directed mutagenesis modifies the A portion (S1 subunit) of the holotoxin, the B portion of the holotoxin or both the A and B portions of the holotoxin.
23. A TOX operon as claimed in claim 21 wherein a single amino acid in the native pertussis holotoxin in removed or replaced.
24. A TOX operon as claimed in claim 21 wherein the single amino acid is (S1) GLU<sup>129</sup> and is removed or is replaced by (S1) GLY<sup>129</sup>, or is (S1) ARG<sup>9</sup> and is replaced by (S1) LYS<sup>9</sup>, or is (S1) ARG<sup>58</sup> and is replaced by (S1) GLU<sup>58</sup>.
25. A TOX operon as claimed in claim 21 wherein multiple amino acids in the native pertussis holotoxin are removed or replaced.
26. A TOX operon as claimed in claim 21 wherein the multiple amino acids are (S1) GLU<sup>129</sup> TYR<sup>130</sup> and are replaced by (S1) GLY<sup>129</sup> PHE<sup>130</sup>, or are (S1) GLU<sup>129</sup>/(S3) TYR<sup>92</sup>LYS<sup>93</sup> and are replaced by (S1) GLY<sup>129</sup>/(S3)ASN<sup>92</sup>ARG<sup>93</sup>.
27. A TOX operon as claimed in claim 21 wherein (S1) ARG<sup>9</sup>, ARG<sup>13</sup>, TRP<sup>26</sup>, ARG<sup>58</sup> and/or GLU<sup>129</sup> is deleted or replaced.
28. A TOX operon as claimed in claim 21 wherein (S1) ARG<sup>9</sup>, ARG<sup>13</sup> and/or GLU<sup>129</sup> is deleted or replaced.
29. A TOX operon as claimed in claim 21 wherein a residual toxicity less than about 0.5% of the toxicity of the native toxin.
30. A TOX operon as claimed in claim 21 wherein the mutant possesses a decreased histamine sensitisation activity.
31. A strain of Bordetella, characterised by having a mutated toxin operon formed by site-directed mutagenesis to remove or replace at least one nucleotide sequence coding for at least one specific amino acid residue which alone or together confer pertussis toxin toxicity and by the ability to express an immunoprotective genetically-detoxified mutant of pertussis holotoxin having a residual toxicity of 1% or less in the absence of wild type toxin.
32. A strain as claimed in Claim 31, characterised by being B. pertussis strain S-3122-2-3, B. pertussis strain S-3122-3-1, B. pertussis strain S-3036-2, B. pertussis strain 2962-2-1 or B. pertussis strain 3-2962-1-2, having ATCC accession number 53833, 53834, 53835, 53836 and 53837, respectively.
33. A strain as claimed in Claim 31, characterised in that said removed or replaced nucleotide sequence codes for (S1) ARG<sup>9</sup>, ARG<sup>13</sup>, TRP<sup>26</sup>, ARG<sup>58</sup> and/or GLU<sup>129</sup>.
34. A strain as claimed in claim 31, characterised in that said removed or replaced nucleotide sequence codes for (S1) ARG<sup>9</sup>, ARG<sup>13</sup> and/or GLU<sub>129</sub>.

35. A strain as claimed in Claim 31, characterised in that said removed or replaced nucleotide sequence codes for a single amino acid in the native pertussis holotoxin.

36. A strain as claimed in Claim 35, characterised in that said single amino acid is (S1) GLU<sup>129</sup> and said  
5 nucleotide sequence is removed or replaced by one coding for (S1) GLY<sup>129</sup>, said single amino acid is (S1) ARG<sup>9</sup> and said nucleotide sequence is replaced by one coding for (S1) LYS<sup>9</sup>, or said single amino acid is (S1) ARG<sup>58</sup> and said nucleotide sequence is replaced by one coding for (S1) GLU<sup>58</sup>.

37. A strain as claimed in Claim 31, characterised in that said removed or replaced nucleotide sequences  
10 code for multiple amino acids in the native pertussis holotoxin.

38. A strain as claimed in Claim 37, characterised in that said multiple amino acids are (S1) GLU<sup>129</sup> TYR<sup>130</sup>  
and said nucleotide sequences are replaced by ones coding for (S1) GLY<sup>129</sup> PHE<sup>130</sup>; or said multiple  
15 amino acids are (S1) GLU<sup>129</sup>/(S3) TYR<sup>92</sup> LYS<sup>93</sup> and said nucleotide sequences are replaced by ones coding for (S1) GLY<sup>129</sup>/(S3) ASN<sup>92</sup> ARG<sup>93</sup>.

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## FIG. 1.

## AMINO ACID SEQUENCE OF RADIO LABELLED PEPTIDES

CYCLE NO.	5	10	15
PEPTIDE A	Ile Leu Ala Gly Ala Leu Ala Thr Tyr Glu Ser *		Tyr Leu Ala
PEPTIDE B	Ile Leu - Gly Ala Leu Ala Thr Tyr Glu Ser *		Tyr Leu Ala
SUBUNIT S1	Ile Leu Ala Gly Ala Leu Ala Thr Tyr Glu Ser Glu Tyr Leu Ala		
RESIDUE NO.	120	125	130

\* UNCHARACTERIZED RADIOACTIVE AMINO ACID FOUND AT CYCLE 12

— NO AMINO ACID WAS IDENTIFIED AT THIS CYCLE



FIG.2.



FIG.2B.

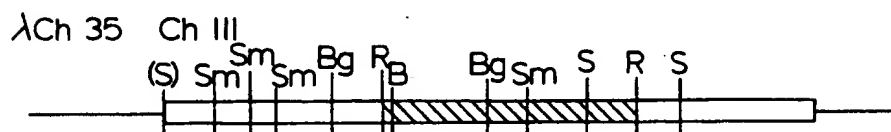


FIG.2C.

$\lambda$ Ch 35 Ch121/Ch411

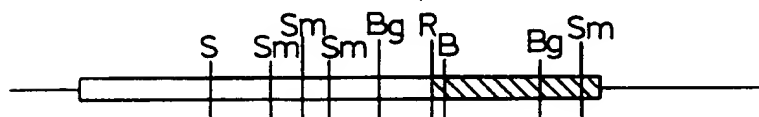


FIG.2D.

$\lambda$ Ch 35 Ch 431

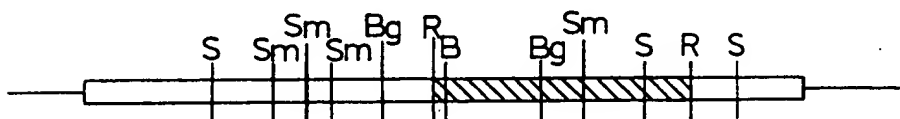


FIG.2E.

$\lambda$ Ch 35 Ch 421

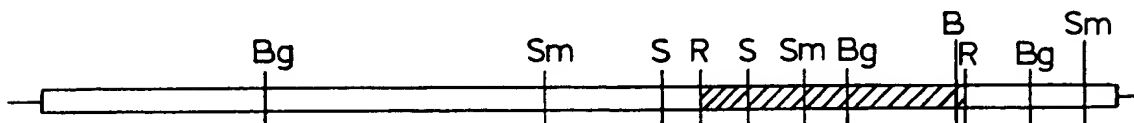


FIG. 3.

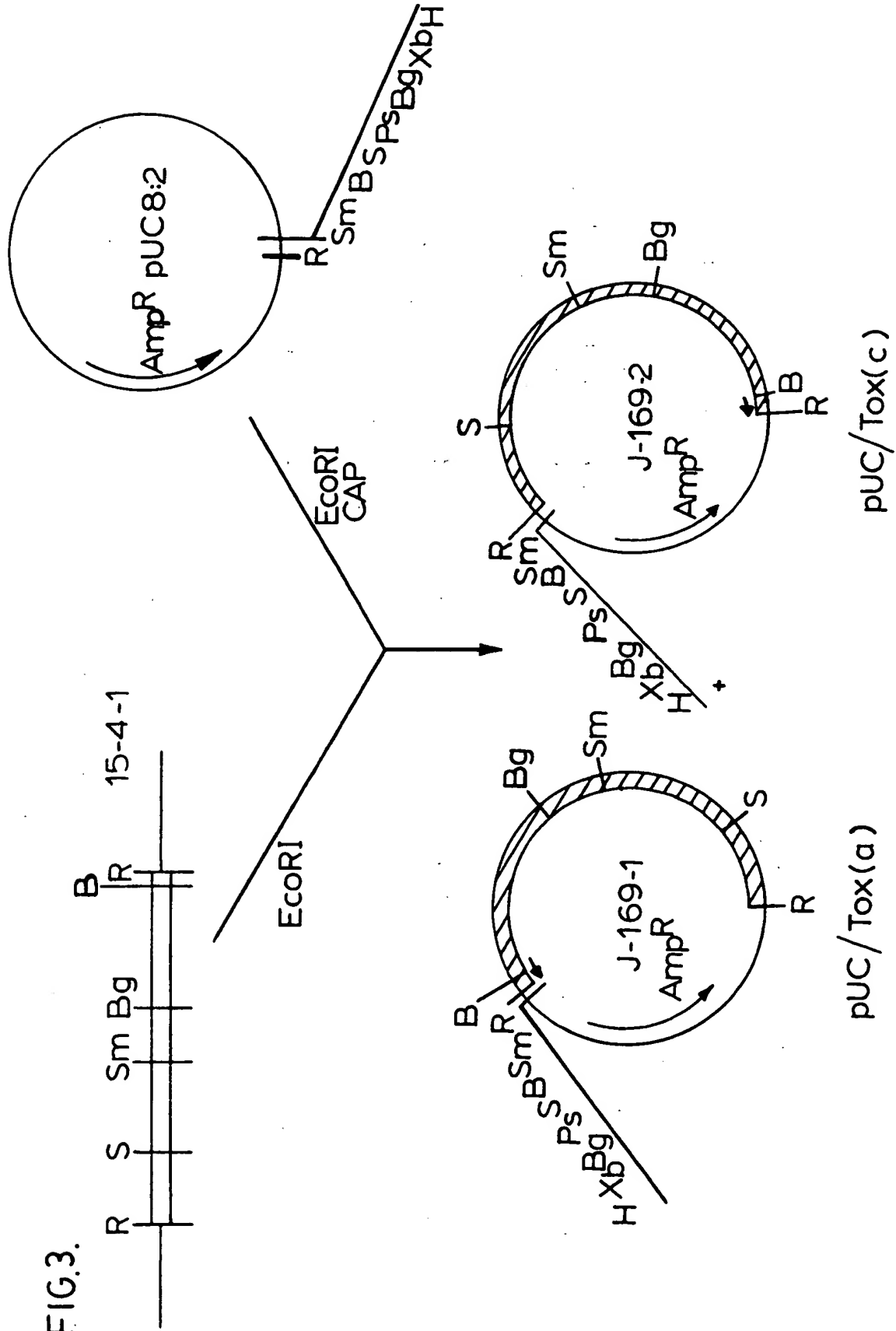


FIG.4A.

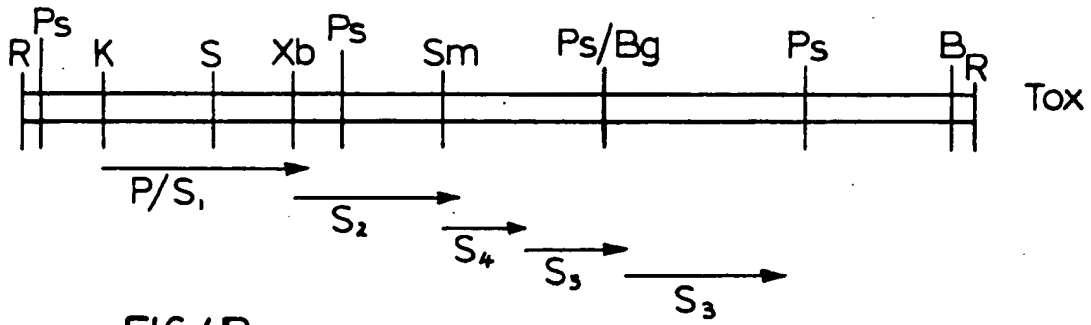


FIG.4B.

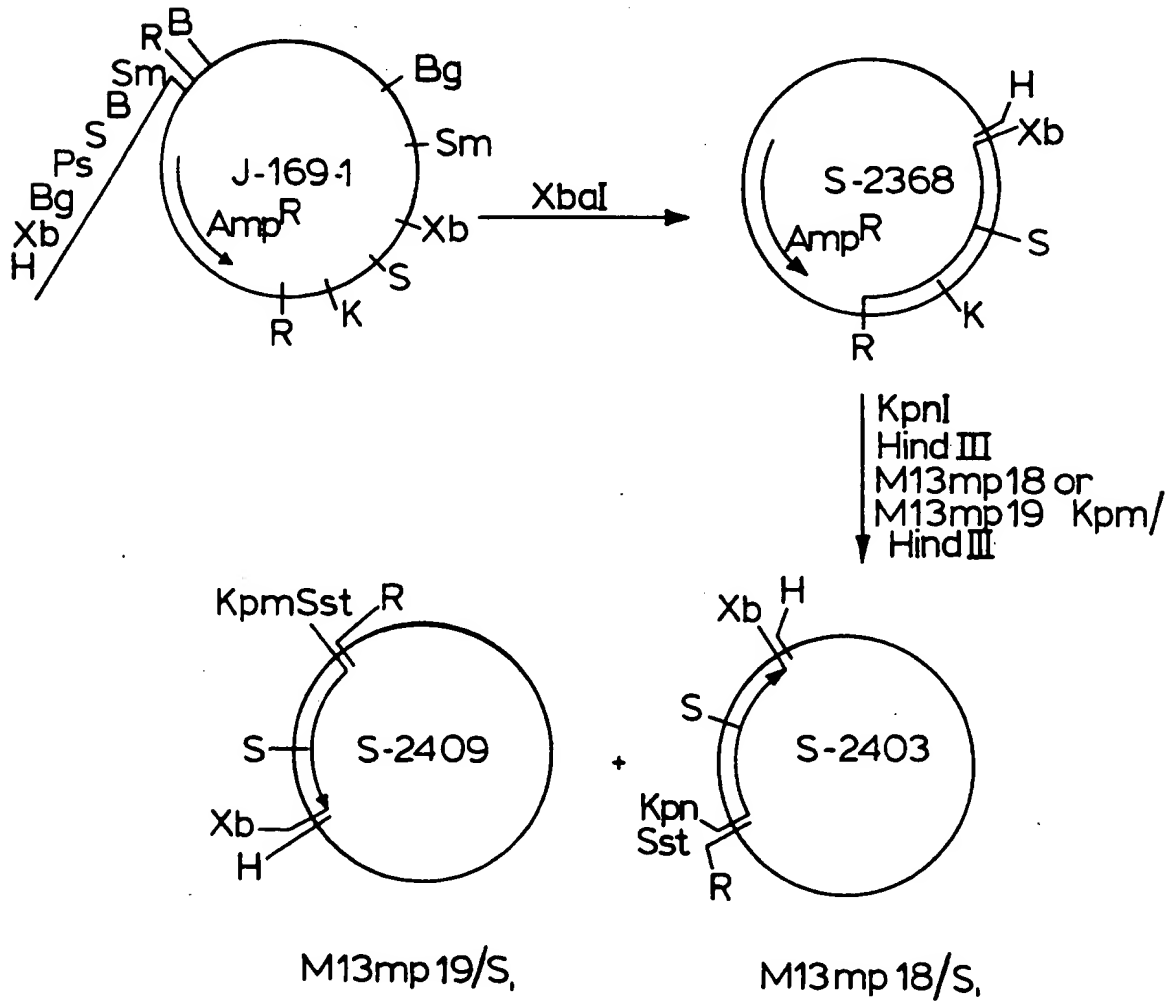


FIG.4C.

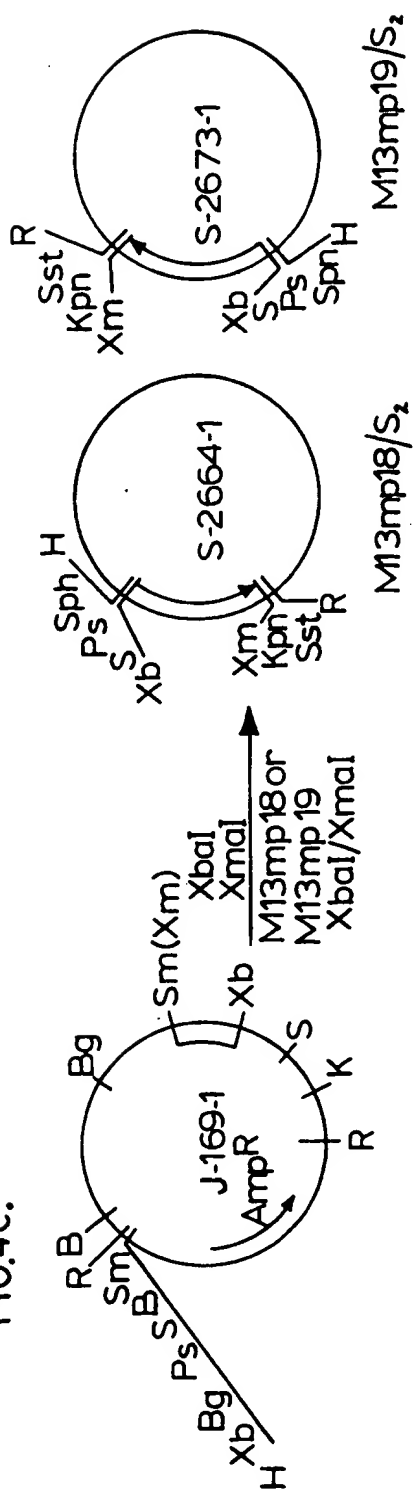


FIG.4D.

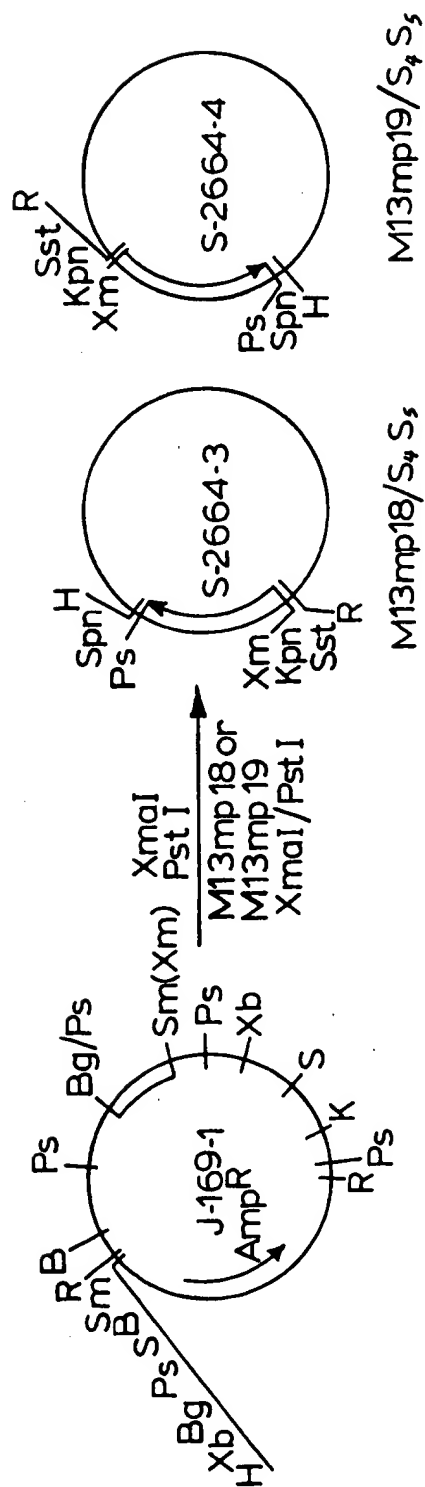
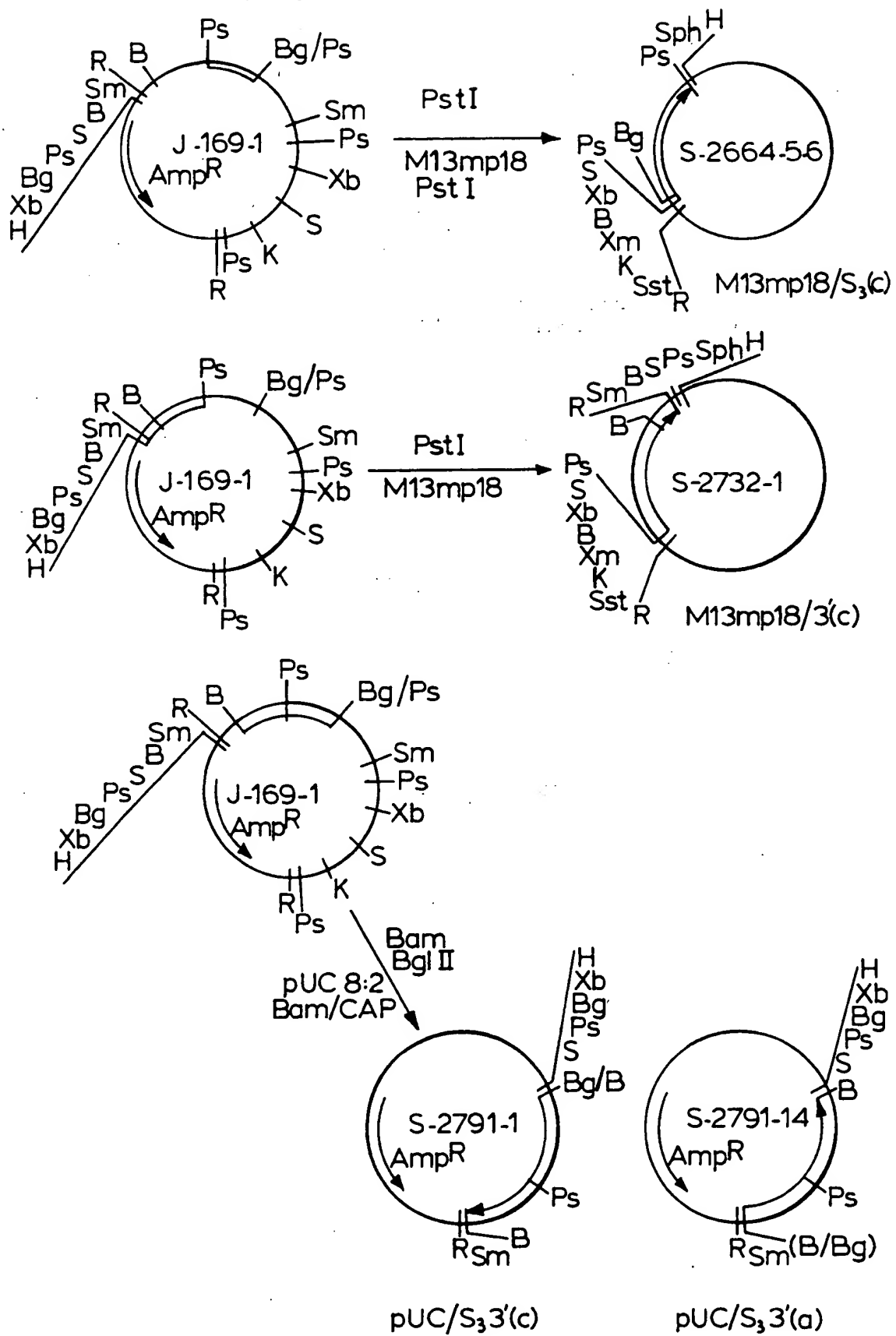


FIG. 4E.



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ref

**and**

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FIG.5(ii).

GAG TAC CTG CGT CAT GTG ACG CCG TGG TCA ATA TTT GCG CTC TAC GAT GGC ACC TAT CTC GGC GGC GAA TAT GGC GGC GTG  
 GLU TYR LEU ARG HIS VAL THR ARG GLY TRP SER ILE PHE ALA LEU TYR ASP GLY THR TYR LEU GLY GLY TYR GLY VAL  
  
 ATC AAG GAC GGA ACA CCC GGC GGC GCA TTC GAC CTG AAA ACG ACG TTC TGC ATC ACC ACG CCG AAT ACG GGT CAA CCC GCA  
 ILE LYS ASP GLY THR PRO GLY GLY ALA PHE ASP LEU LYS THR THR PHE CYS ILE MET THR THR ARG ASN THR GLY GLN PRO ALA  
  
 ACG GAT CAC TAC TAC ACG AAC AAT ACC GGC ACT CCG CTG CTC TCC ACG ACC AAC ACG ACG CTA TGC GCG GTC TTC GTC AGA ACG  
 THR ASP HIS TYR TYR SER ASN VAL THR ALA THR ARG LEU LEU SER SER THR ASN SER ARG LEU CYS ALA VAL PHE VAL ARG SER  
  
 GGG CAA CCG GTC ATT GGC GGC TGC ACC ACG CCG TAT GAC GGC AAG TAC TGG ABC ATG TAC ACG CCG CTG CCG AAA ATG CTT TAC  
 GLY GLN PRO VAL ILE GLY ALA CYS THR SER PRO TYR ASP GLY LYS TYR TRP SER MET TYR SER ARG LEU ARG LYS MET LEU TYR  
  
 CTG ATC TAC GTG GGC ATC TCC GTC CAC GTC CAT GTG ACG AAG GAA CAG TAT TAC GAC TAT GAG GAC GCA ACG TTC GAG  
 LEU ILE TYR VAL ALA GLY ILE SER VAL ARG VAL HIS VAL SER LYS GLU GLU TYR TYR ASP TYR GLU ASP ALA THR PHE GLU  
  
 ACT TAC GGC CTT ACC GGC ATC TCC ATC TGC AAT CCT GGA TCA TCC TTA TGC TGACGCTTCCCACTCGAACCCGCCGCCGACGAGGCG  
 THR TYR ALA LEU THR GLY ILE SER ILE CYS ASN PRO GLY SER LEU CYS  
  
 CCCCCGCGGTCCGCG GTG CCG GGC CTG GCG TGG TTG CTG GCA TCC GGC GCG ATG ACG CAT CTT TCC CCC GGC CTG GGC GAC GTT CCT  
 fMET ARG ALA LEU ALA TRP LEU LEU ALA SER GLY ALA MET THR HIS LEU SER PRO ALA LEU ALA ASP VAL PRO  
 34  
 TAT GTG CTG GTG AAG ACC AAT ATG GTG GTC ACC ACG GTA GGC ATG AAG CCG TAT GAA GTC ACC CCG ACG CCG ATG CTG GTC TGC  
 TYR VAL LEU VAL LYS THR ASN MET VAL VAL THR SER VAL ALA MET LYS PRO TYR GLU VAL THR PRO THR ARG MET LEU VAL CYS  
  
 GGC ATC GGC GGC AAA CTG GGC GGC GGC ACG ACG CCG GAC GCG CAC GTG TGC TTC GGC AAG GAT CTC AAG CGT CCC  
 GLY ILE ALA ALA LYS LEU GLY ALA ALA SER SER PRO ASP ALA HIS VAL PRO PHE CYS PHE GLY LYS ASP LEU LYS ARG PRO  
  
 GGC ABC AGT CCC ATG GAA GTC ATG TTG CCG GGC GTC TTC ATG CAA CAA CCG CCG CTG CCG ATG TTT CTG GGT CCC AAG CAA CTC  
 GLY SER SER PRO MET GLU VAL MET LEU ARG ALA VAL PHE MET GLN GLN ARG PRO LEU ARG MET PHE LEU GLY PRO LYS GLN LEU  
  
 ACT TTC GAA GGC AAG CCC GCG CTC GAA CTG ATC CCG ATG GTC GAA TGC ACG GGC AAG CAG GAT TGC CCC TGA AGCGCAACCCC ATG  
 THR PHE GLU GLY LYS PRO ALA LEU GLU LEU ILE ARG MET VAL GLU CYS SER GLY LYS GLN ASP CYS PRO  
 fMET  
 35  
 CAT ACC ATC GCA TCC ATC CTG TTG TCC GTG CTC GGC ATA TAC ACG CCG GGT GAC GTC GGC GGC TTG CCG ACC CAT CTG TAC AAG  
 HIS THR ILE ALA SER ILE LEU LEU SER VAL LEU GLY ILE TYR SER PRO ALA ASP VAL ALA GLY LEU PRO THR HIS LEU TYR LYS  
  
 AAC TTC ACT GTC CAG GAG CTG GGC TTG AAA CTG AAG GGC AAG AAT CAG GAG TTC TGC CTG ACC GGC TTC ATG TCG GGC AGA ACG  
 ASN PHE THR VAL GLN GLU LEU ALA LEU LYS LEU LYS GLY LYS ASN GLN GLU PHE CYS LEU THR ALA PHE MET SER GLY ARG SER  
  
 CTG GTC CCG GCG TGC CTG TCC GAC GCG GGA CAC GAG CAC ACG TGG TTC GAC ACC ATG CTT GGC TTT GGC ATA TCC GCG TAT  
 LEU AVL ARG ALA CYS LEU SER ASP ALA GLY HIS GLU HIS ASP THR TRP PHE ASP THR MET LEU GLY PHE ALA ILE SER ALA TYR  
  
 GCG CTC AAG ACG CCG ATC GCG CTG ACG GTG GAA GAC TCG CCG TAT CCG GGC ACT CCC GGC GAT CTG CTC GAA CTG CAG ATC TGC  
 ALA LEU LYS SER ARG ILE ALA LEU THR VAL GLU ASP SER PRO TYR PRO GLY THR PRO GLY ASP LEU LEU GLN ILE CYS

CCG CTC AAC GGA TAT TGC GATGAAACCCCTCCGAGGTTTCGACGTTTCGCGCAATCCGCTTGAGACGATCTTCGCGCCCTGGTTCCATTCCGGGAACACCGCAAC  
PRO LEU ASN GLY TYR CYS GLU

ATG CTG ATC AAC AAC AAG AAG CTG CTT CAT CAC ATT CTG CCC ATC CTG GTG CTC GCC CTG GGC ATG CGC ACG GCC CAG GCC

5

VAL ALA PRO GLY ILE VAL ILE PRO PRO LYS ALA LEU PHE THR GLN GLN GLY GLY ALA TYR GLY ARG CYS PRO ASN GLY THR ARG  
GTT GCG CCA GGC ATC GTC ATC CCG CCG AAG GCA CTG TTC ACC CAA CAG GGC GGC TAT GGA CCG TGC CCG AAC GGA ACC CCG

CCC TTG ACC GTG GCC GAA CTG CCG CCG AAC GCC GAA TTG CAG ACG TAT TTG CCG CAG ATA ACG CCC GCG TGG TCC ATA TAC GGT

ALA 29 LEU 30 THR 31 VAL 32 ALA 33 GLU 34 LEU 35 ARG 36 GLY 37 ASN 38 ALA 39 GLU 40 LEU 41 GLN 42 THR 43 TYR 44 LEU 45 ARG 46 GLN 47 ILE 48 THR 49 PRO 50 GLY 51 IRP 52 SER 53 ILE 54 TYR 55 GLY 56

[illegible]

ACT TTC TGC ATC ACG ACC ATA TAC AAG ACC GGG CAA CCG GCT GCG GAT CAC TAC ACG AAG GTC ACG GCC ACG CGC CTC

THR	85	PHE	86	CYS	87	ILE	88	THR	89	THR	90	ILE	91	THR	92	THR	93	LYS	94	THR	95	GLY	96	PRO	97	ALA	98	ALA	99	ASP	100	His	101	TYR	102	103	104	SER	105	LYS	106	VAL	107	THR	108	ALA	109	THR	110	ARG	111	LEU	112
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[illegible]

AGG TAC AGA GAC ATG TAC GAC GCG CTG CCG CSC CTG CTG TAC ATG ATC TAT ATG TCC GGC CTT GGC GTT CAC GTC AGC

Year	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100																																																		
Age	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300

[illegible]

TCG ATA TGC TGAACCCGCGCTGGATCTGTTGCGCTGTCCATGTTTTTCTTGACGGATACCGGAAATGAATCCCTTGAAGACTTGAAGACATCGCTACGCGCCCT

197 198 199

666CCCTTCA T65CA6CCT6C2CACCT6 TT6TC6CCCA C65TC6CC6ACCTTCG6CCCA665C666C58C666T6CA6C6C6GTCAACACCTTCA T66C6G6A6CA T6766TC6TACT6C  
C66C66C6C6GTCA6T666CCAC66T6ACCA TCG6CA TAA TCT666C666TACAA6CT6CT6T TTTCC66CAC66CCCA T6T6CT66AC6T666T6C6A6T66T6CT66C6G66A6CT6C

CGTACCGCCACGCCGCTGGCCGTGTGACAGGSCACCAATTGCCCTGCTGGGCACTGGTTCAECA TGCCCTTTC TGCCCTTGT TCCCGTGCA TGTCTGSCCA TCGGGA TC

ATGATCCGGCCGATGACCAACAGTTCGCGCTGATC



FIG.6A.

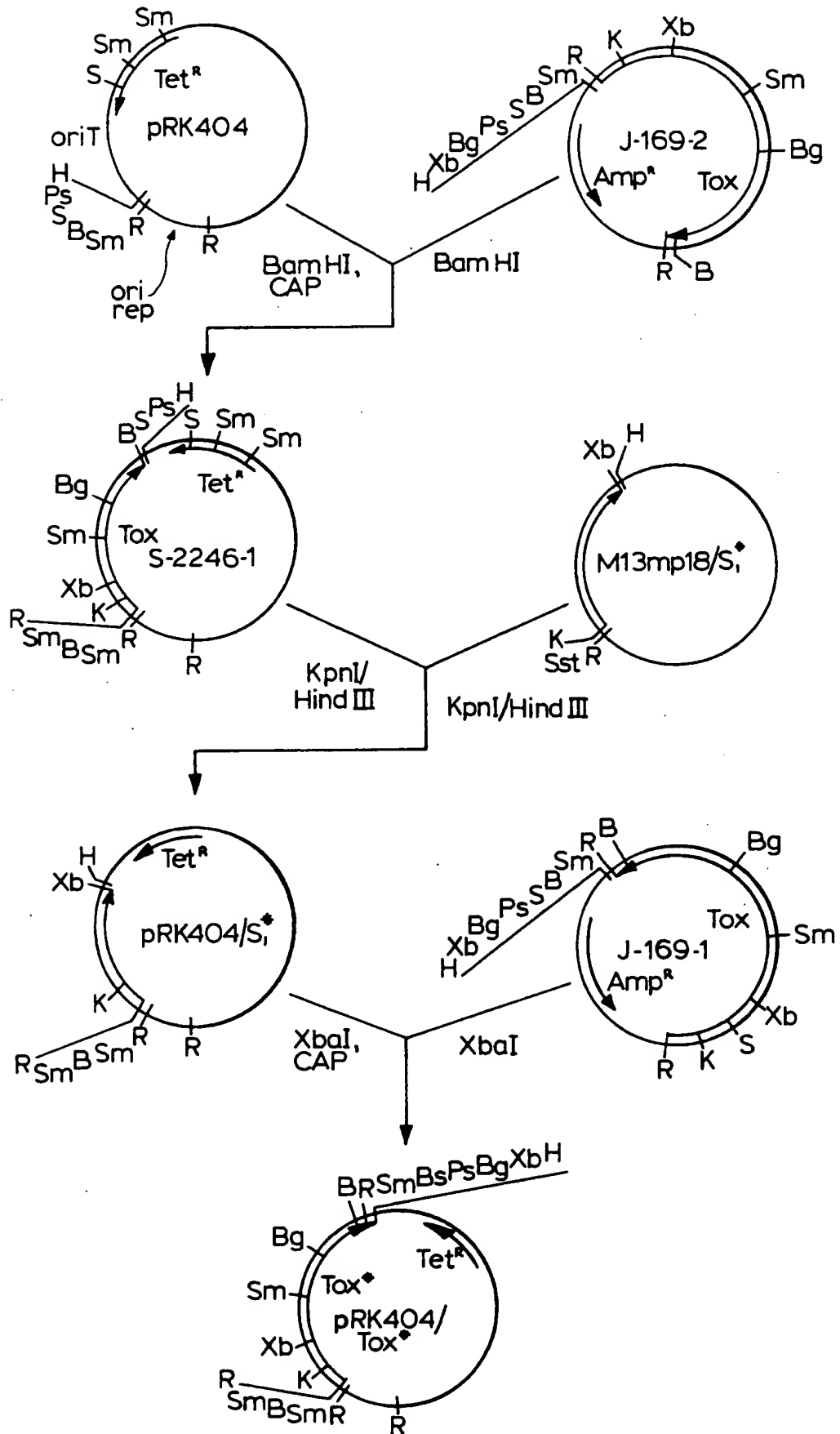


FIG.6B.

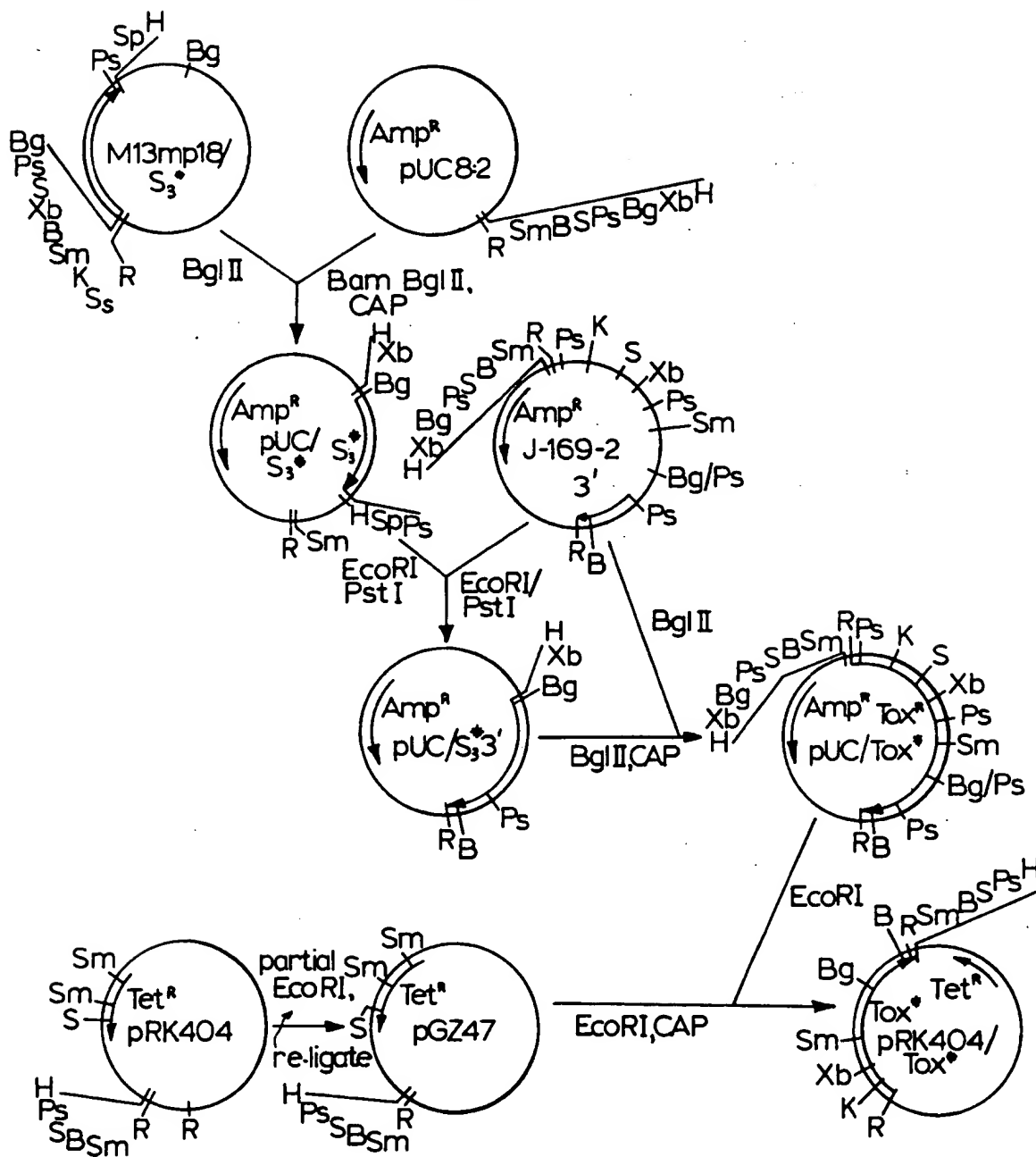
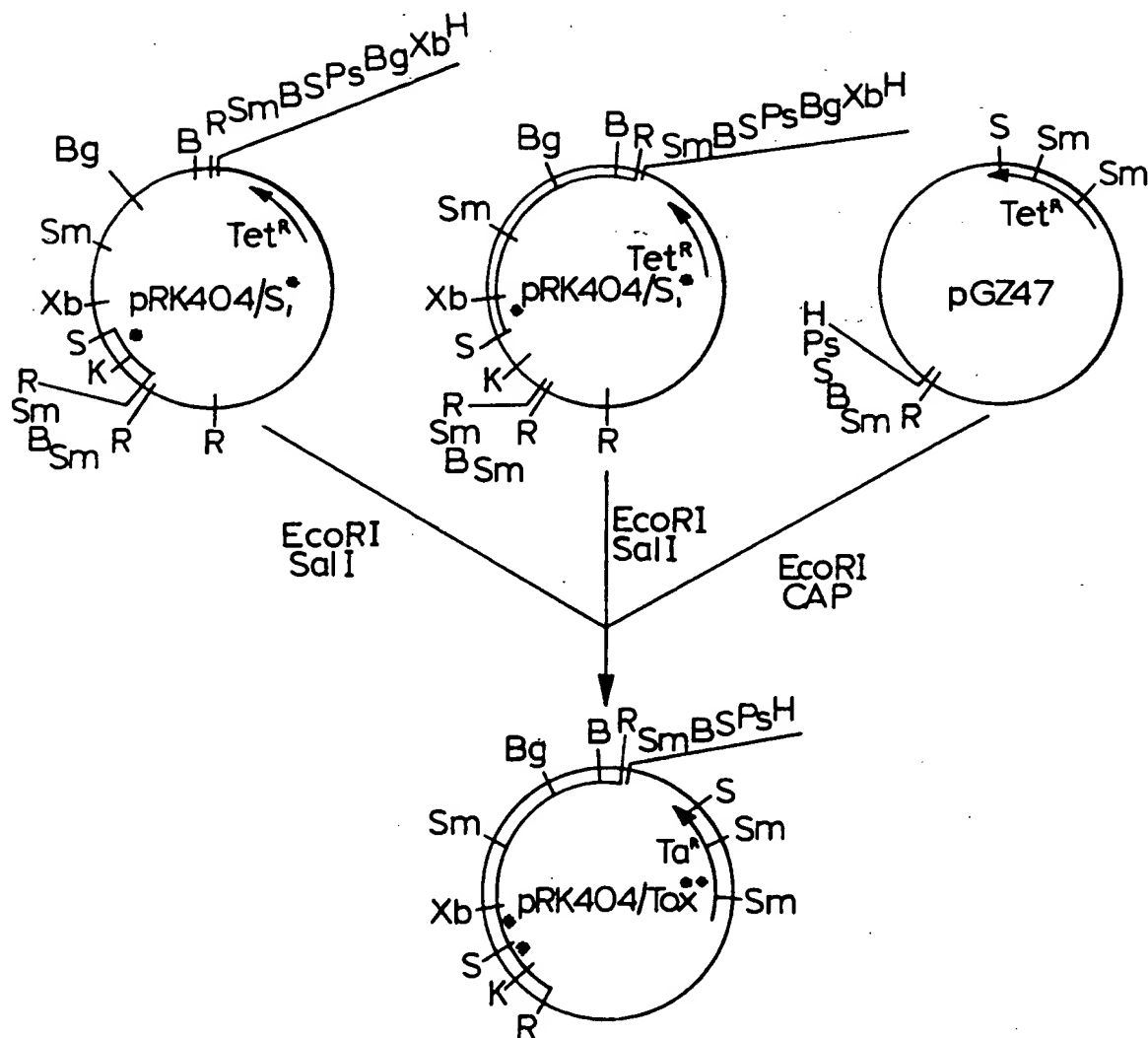


FIG.6C.



\* DENOTES THE LOCATION OF MUTATIONS IN THE S<sub>1</sub> GENE

FIG. 7.

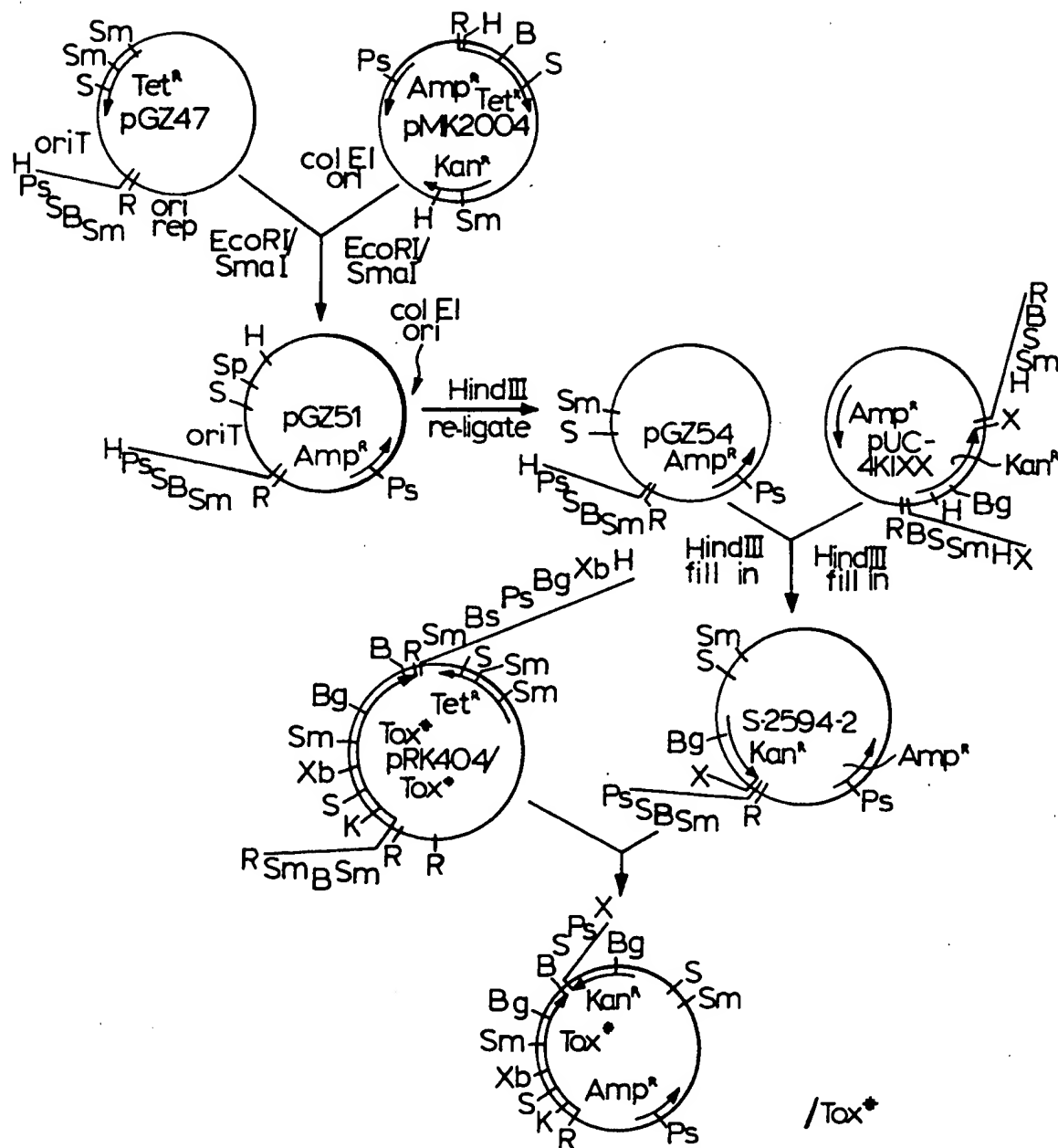


FIG.8A.

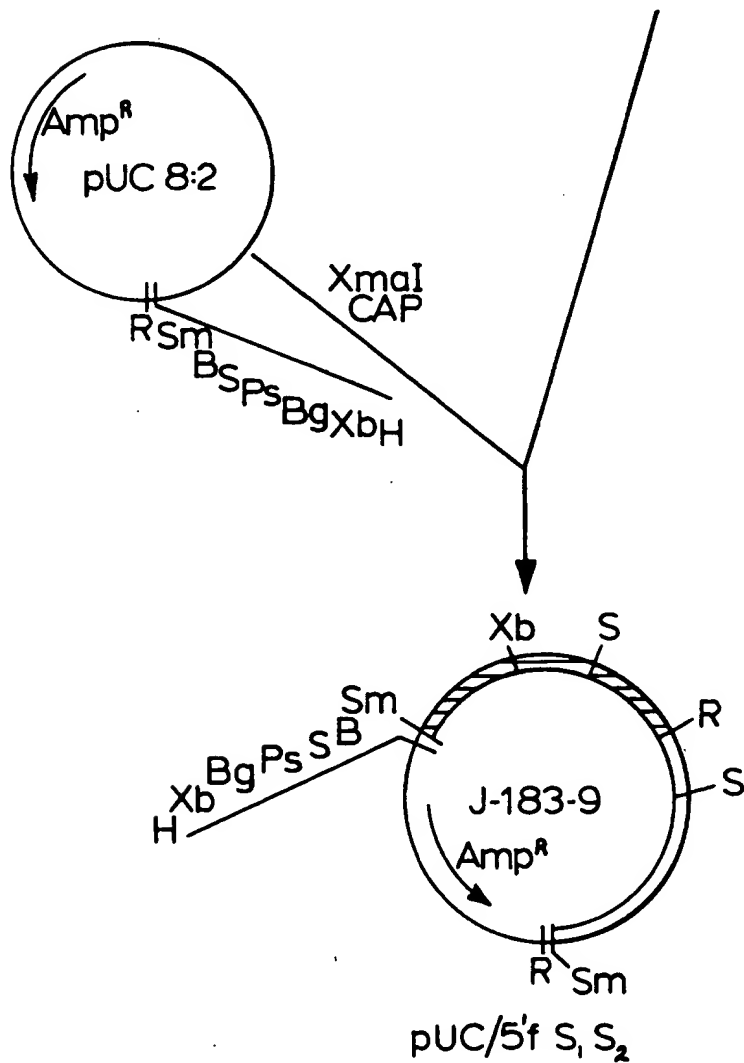
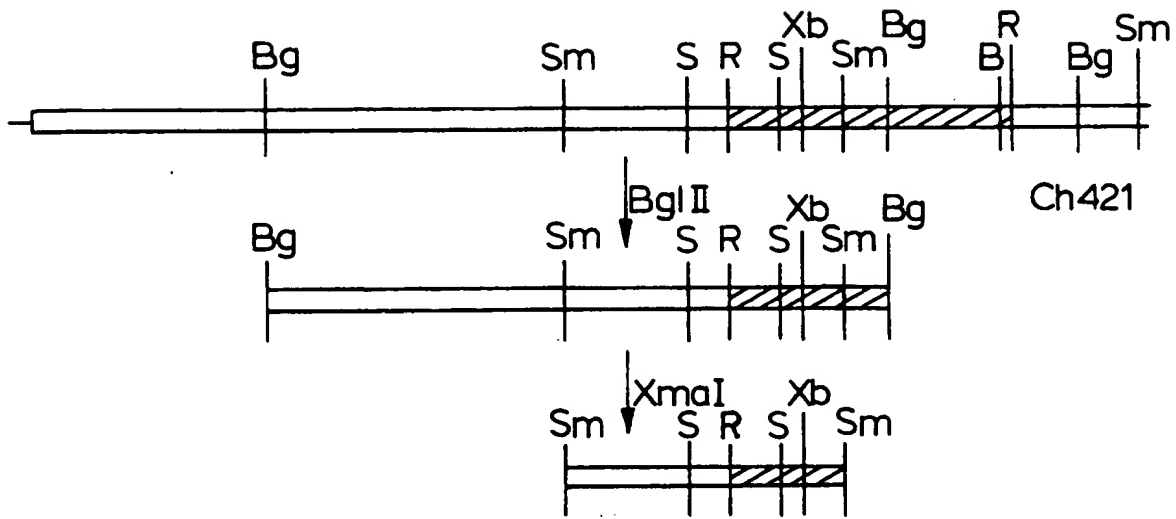


FIG.8B.

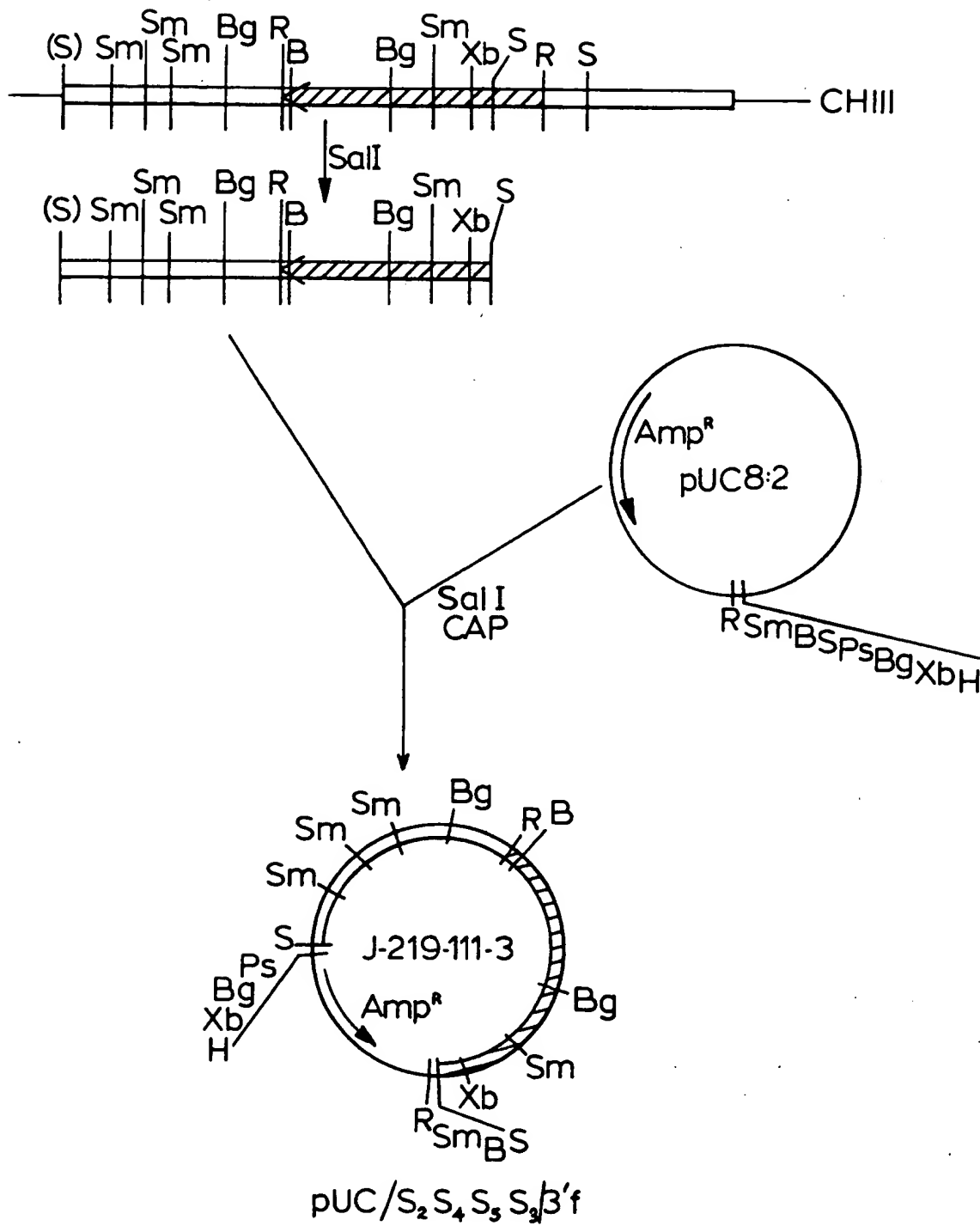


FIG.8C.

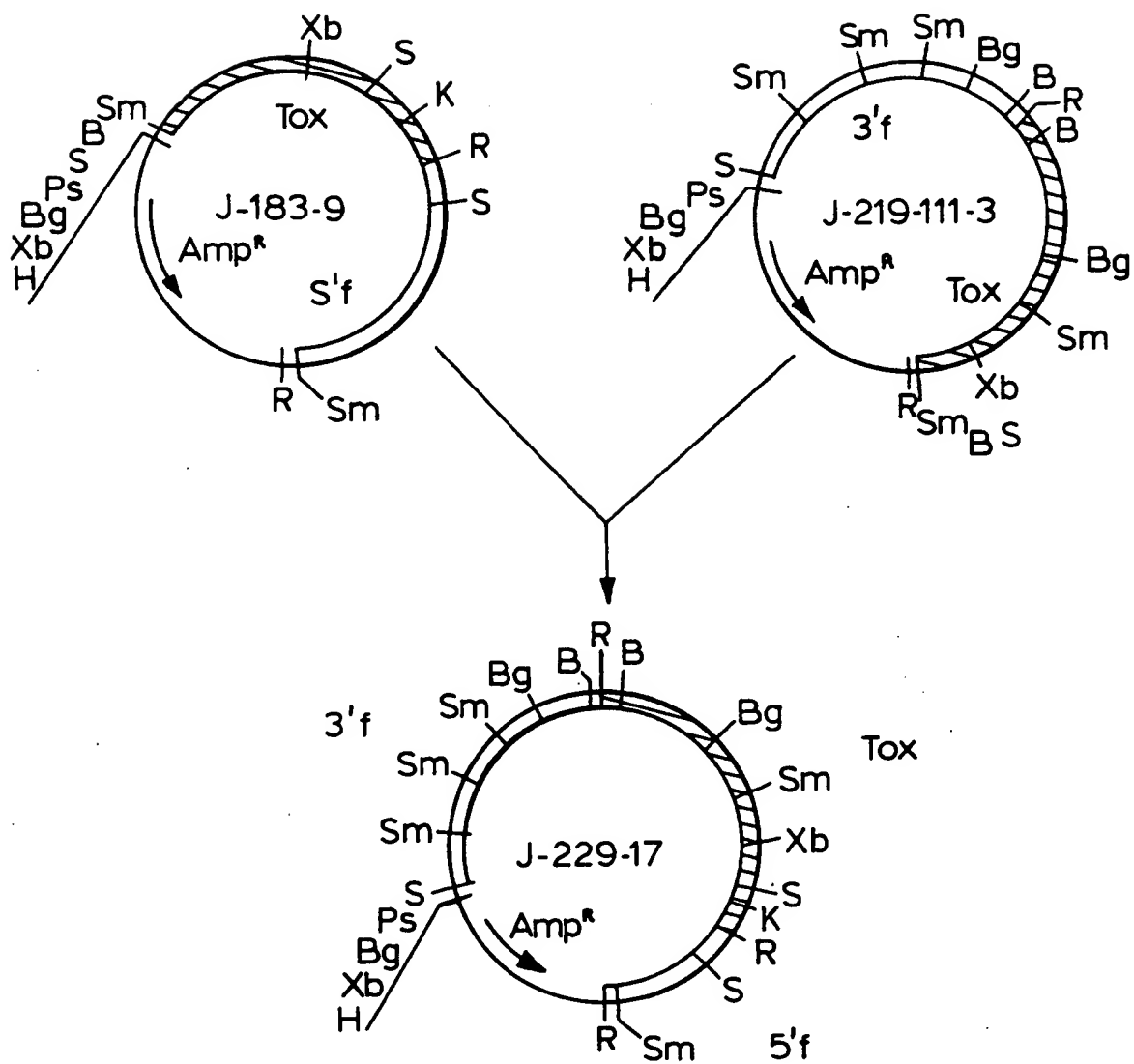


FIG. 9A.

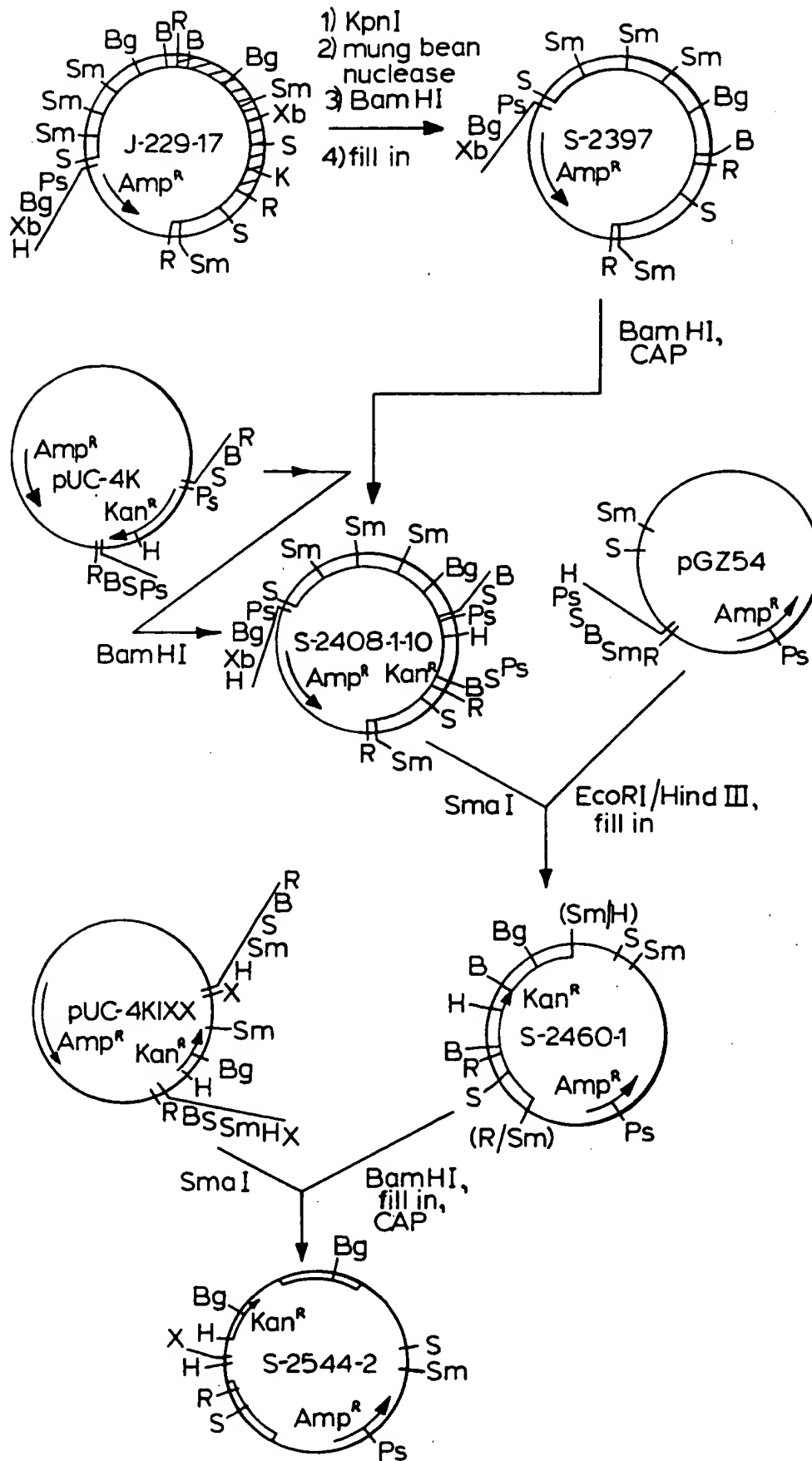
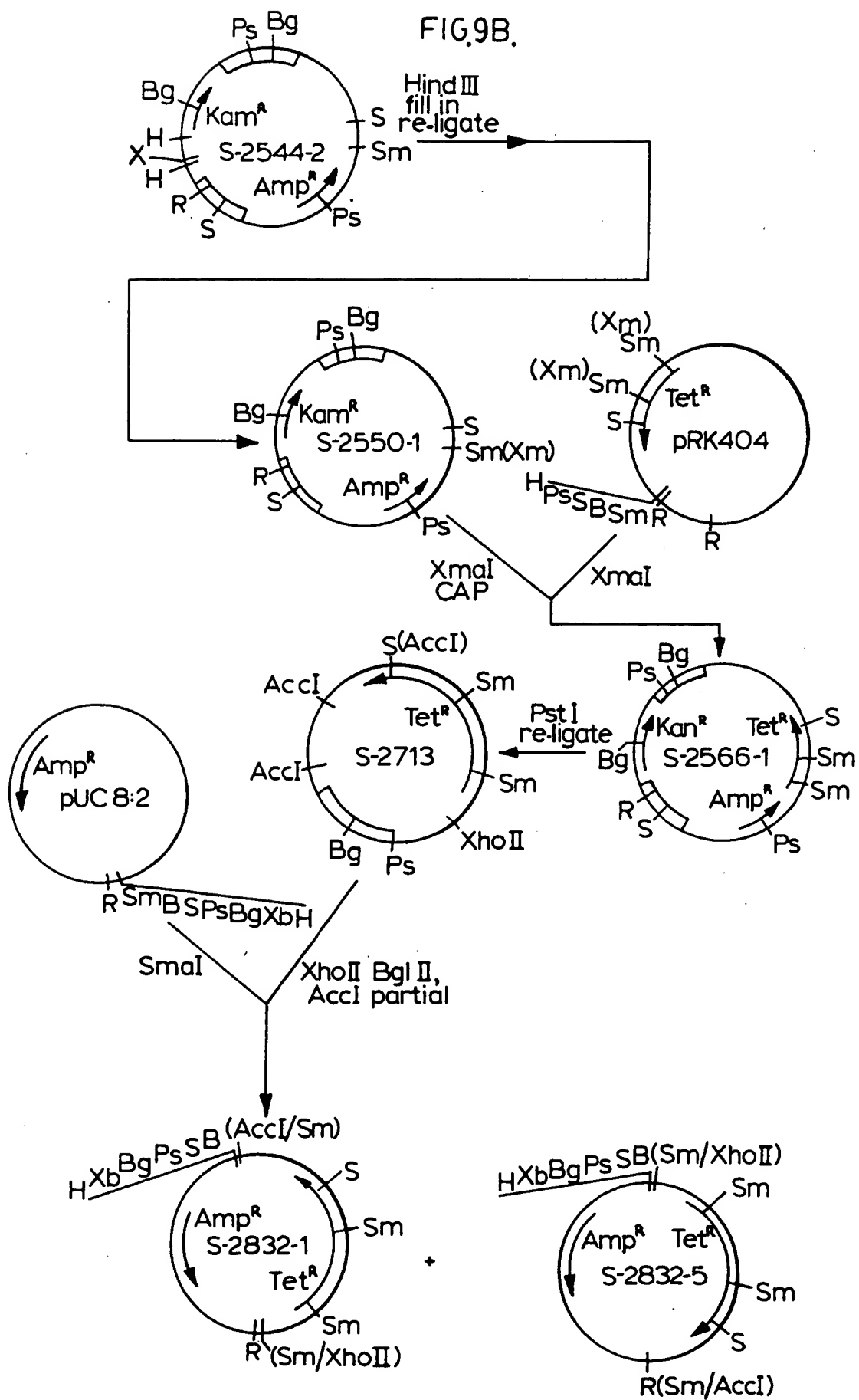




FIG.9B.



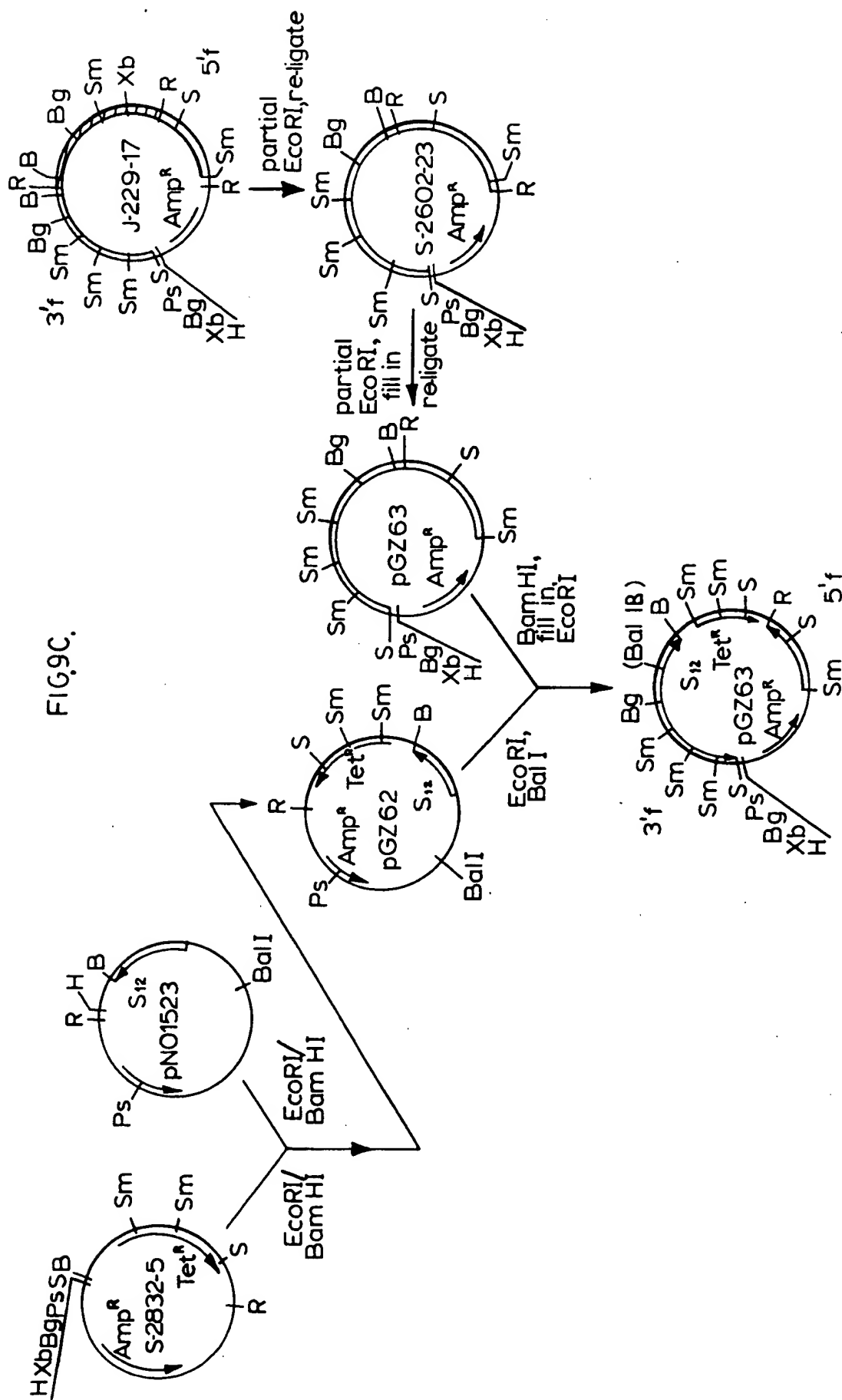
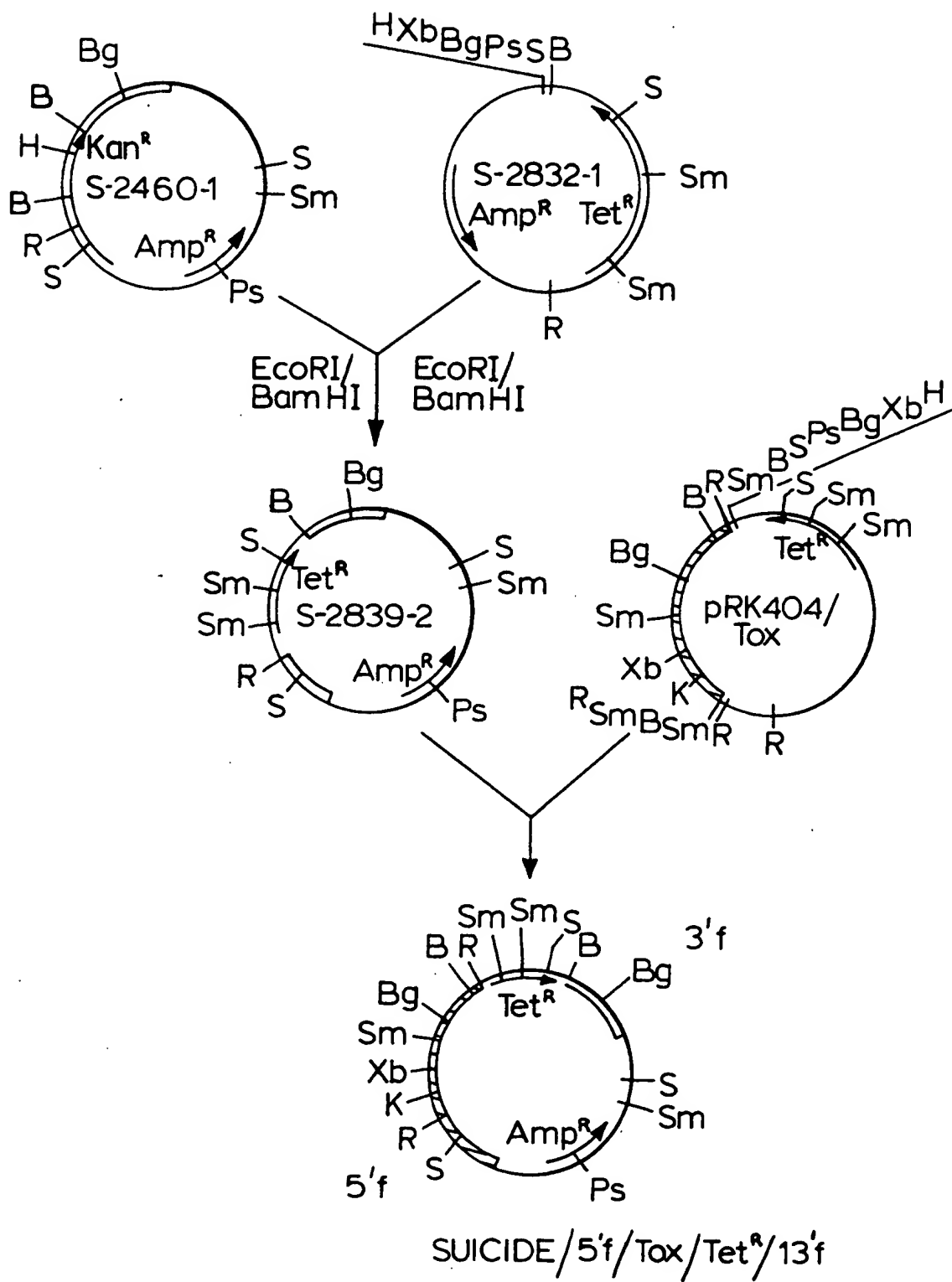


FIG.10.





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# EUROPEAN SEARCH REPORT

Application Number  
EP 95 11 1215

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. CL 4)
A	SCIENCE , vol. 232, - 1986 pages 1258-1264, C. LOCHT ET AL. 'Pertussis toxin gene: nucleotide sequence and genetic organisation' * the whole document * ---	1	C12N15/00 A61K39/10
A	PROC. NATL. ACAD. SCI. USA, vol. 83, - 1986 pages 4631-4635, A. NICOSIA ET AL. 'Cloning and sequencing of the pertussis toxin genes: operon structure and gene duplication' * the whole document * ---	1	
A	INF. IMMUN., vol. 55, no. 4, - 1987 pages 963-967, A. NICOSIA ET AL. 'Expression and immunological properties of the 5 subunits of pertussis toxin' * the whole document * ---	1	
X,P	SCIENCE, vol. 242, - 1988 pages 72-74, W. BURNETTE ET AL. 'Pertussis toxin S1 mutant with reduced enzyme activity and a conserved protective epitope' * table 1 * ---	1-4, 7-15, 18-24, 27-36	TECHNICAL FIELDS SEARCHED (Int. CL 4)  C07K C12N A61K
E	EP-A-0 322 533 (SCLAVO)  * the whole document * ---	1-5, 7-16, 18-25, 27-37	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 9 October 1995	Examiner Skelly, J
<b>CATEGORY OF CITED DOCUMENTS</b> X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document  T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document			



European Patent  
Office

# EUROPEAN SEARCH REPORT

Application Number  
EP 95 11 1215

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.4)
E	EP-A-0 306 318 (AMGEN INC.)  * the whole document * ---	1-4, 7-15, 18-24, 27-36	
X,P	INF. IMMUN., vol. 56, no. 8, - 1988 pages 1934-1941, J. BARBIERI ET AL 'APD-ribosyl transferase mutations in the catalytic S1 subunit of pertussis toxin' * the whole document * -----	1-4, 7-15, 18-24, 27-36	
			TECHNICAL FIELDS SEARCHED (Int.Cl.4)
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 9 October 1995	Examiner Skelly, J
<b>CATEGORY OF CITED DOCUMENTS</b> X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document  T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ..... & : member of the same patent family, corresponding document			

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